REMARKS

Status of the Claims

Claim 111 is allowed. Claims 63-90, 93, 94, 97-111, and 113-125 are currently pending and at issue.

Claims 83, 87, 89, 93, 97, 99, 101, 105, 109, and 116-125 have been amended to delete the "ATCC 46032" questioned by the Examiner.

Claims 63 and 64 have been amended to recite "a wild-type galactose oxidase having the sequence of SEQ ID NO:10 wherein the amino acid D at position 537 of SEQ ID NO: 10 is N in the wild-type." This amendment follows the Examiner's suggestion regarding the reference wild-type and the position of each mutated amino acid in that wild-type sequence.

The phrase "and wherein the variant has improved D-galactose oxidastion activity as compared to the wild-type galactose oxidase" has been deleted from claim 84 and added to claim 83.

Claims 83, 87, 89, 93, 97, 99, 101, 103, 105, 107, and 109 have been amended as per claims 63 and 64.

Claims 116-125 have been amended to delete "*D. Dendroides.*"

No new matter has been added by these amendments.

Claim Rejections

35 USC 112, first paragraph

Claims 83-90, 93-94, 97-110, and 115, 125 previously referred to a microorganism deposit: ATCC 46032. These claims were rejected under 35 USC 112, first paragraph, for lack of enablement. The Examiner stated that the microorganism required to practice the claimed invention

is not known and readily available to the public or obtainable by a repeatable method set forth in the specification.

Claims 83, 87, 89, and 101 have been amended to delete the phrase "from ATCC 46032". Claims 84-86, 88, 90, and 102 depend from these amended claims. Claims 93, 97, 99, 105, 109, and 125 have also been amended to delete the phrase "of ATCC 46032". Claims 94, 98, 106, and 110 depend from these amended claims. These claims have been further amended to refer to a specific wild-type amino acid sequence that is then mutated, as provided in the specification. This is SEQ ID NO: 10, which is a wild-type galactose oxidase protein of *D. Dendroides* when the amino acid D at position 537 of SED ID NO: 10 is N. See specification, at page 47, Table 4, and Figure 17A, showing the sequence of SEQ ID NO: 10 and indicating that the mutant has only the amino acid substitution N537D in the wild-type amino acid sequence.

Accordingly, a microorganism deposit is not needed to support the invention as claimed, and references to "ATCC 4602" have been removed.

Further, disclosed gaoA gene encoding the wild-type galactose oxidase protein from *D. dendroides* is sufficiently described and enabled. Likewise for the corresponding amino acid sequence. See, e.g., Amgen, Inc. v. Chugai Pharma Co., 927 F.2d 1200, 1211 (Fed. Cir. 1991). Wild-type *D. dendroides* galactose oxidase is also well-known in the art, with a sequence that has been published and is readily accessible to one of ordinary skill in the art. Support exists in the specification and in the art as follows.

The galactose oxidase protein and gaoA gene are well known in the art

Support can be found in art cited in the present specification:

> The gene of the galactose oxidase has been cloned (110 [McPherson MJ et al., JBC 267(12) April 25, pp 8146-8152, 1992]) and expressed in *Escherichia coli* (127). (p 26 lines 22-23)

The McPherson paper (See Exhibit A), incorporated by reference in the instant application (p. 2, 6-8), clearly sets forth the gaoA gene sequence coding for galactose oxidase of *Dactylium dendroides* to be used in the present invention. The published reference indicates that

> the gaoA nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number(s) M86819. (p. 8146 c1 footnote)

Futher, the editorial policy of the JBC requires that the author of the publication honor any reasonable request by qualified investigator for cell lines or DNA clones. See, http://www.jbc.org/misc/edpolicy.shtml, (Exhibit F, page 1 paragraph 7).

The known wild-type sequence is the same as the wild-type disclosed by SEQ ID NO: 10 of the specification, which shows the N537D mutant of this wild-type. See specification, at page 47, Table 4, and Figure 17A.

The galactose oxidase protein and gaoA gene sequences are readily accessible.

A text search of the (National Center For Biotechnology Information; http://www.ncbi.hlm.nih.gov) NCBI protein database for "galactose oxidase *Dactylium dendroides*" returned 11 hits, including AA16228, "galactose oxidase", which references the McPherson JBC paper (See Exhibit B). A text search of the NCBI entrez nucleotide database for "galactose oxidase *Dactylium dendroides*" returned 5 hits, including M86819, "Dactylium dendroides ...galactose oxidase monomer (gaoA) gene, complete cds", which references the McPherson JBC paper (See Exhibit C). Clicking on the previously described hyperlinks on the web pages depicted in Exhibit B and C connect the artisan to the galactose oxidase nucleotide and protein sequence information (See Exhibit E). Thus, even without SEQ ID NO: 10, an ordinary skilled artisan could easily obtain the galactose oxidase protein and gene sequences necessary to perform the present invention.

The galactose oxidase protein and gaoA gene sequences in the present application were derived from the gaoA gene cloned by McPherson and can be correlated to the Genbank accession number.

Note also that the plasmid containg the gaoA gene in the present application can be directly correlated to the publicly available Genbank accession number.

1) The specification describes the gaoA gene encoding the galactose oxidase protein as cloned from plasmid pGAO-010 from Kuramitsu plasmid pR3. This is described in the following excerpts from the specification.

> Plasmids were constructed to *express* galactose oxidase gene (gao) from *Fusarium* ssp. as described below. (p. 34, lines 24-25)
>
> As shown in FIG. 8, **plasmid pGAO-010 expressing GAO was made using plasmid pR3. Plasmid pR3 contains the gene for** mature **galactose oxidase (GAO)** fused to the 5' end of the *lacZ* fragment, and was obtained from Dr. Howard K. Kuramitsu (Dept. of Oral Biology, State University of New York, Buffalo, NY). (p. 35, line 11) (emphasis added)

2) Kuramitsu's laboratory cloned the gaoA gene in their plasmid pR3 from the McPherson's pGA09 plasmid. This is described in reference number #127 of the present application (See Exhibit D, Lis, M., and Kuramitsu, H.K. (1997) Galactose oxidase-glucan binding domain fusion proteins as targeting inhibitors of dental plaque bacteria., Antimicrob. Agents Chemother., 41(5), 999-1003), excerpted below.

> A 178-bp **EcoRI-BamHI DNA fragment** (Fig. 1A) **from plasmid pGAO9** (10[McPherson MJ JBC 267: 8146 1992]), **containing the 5' end of the sequence coding for mature GAO**, was subcloned into vector pUC118. One of the two DdeI sites on this fragment, located downstream from the codon adjacent to a cleavage site of the leader peptide, was chosen as a point of fusion with the 5' end of the lacZ fragment. The 800-bp DdeI fragment consisting of the subcloned gao sequence and part of pUC118 was excised and, after the ends were filled in with the Klenow fragment of DNA polymerase I, inserted into the SmaI site of vector pUC118Nar (a derivative of pUC118 lacking the NarI site). The resulting plasmid, pO5, was digested with NarI and XbaI, and a **NarI-XbaI DNA fragment from pGAO9, containing**

> **the 3' end of the gene, was inserted, generating plasmid pR3** (Fig. 1B). (emphasis added)

3) McPherson's paper describes the original cloning of the gaoA gene encoding the galactose oxidase protein. McPherson is reference #110 of the present application (See Exhibit A)[1]. pGA09 in the reference contains the gaoA sequence which encodes the galactose oxidase protein, deposited to GenBank under reference number M86819.

> The **nucleotide sequence**(s) reported in **this** paper has been submitted to the **GenBank**™/EMBL Data Bank with accession number(s) **M86819**. (p. 8146 c1 footnote) (emphasis added)
>
> The **sequence of 3466 nucleotides** of **pGAO9** is shown in Fig. 3. (p. 8150, c1) (emphasis added)
>
> Galactose oxidase (EC 1.1.3.9) is **secreted** by the Deuteromycete fungus ***Dactylium dendroides***... (p. 8146, c2, par 1) (emphasis added)

4) The GenBank accession number M86819 contains a reference to the amino acid sequence AAA16228 (Exhibit E), thus providing a connection between the galactose oxidase gene and protein described in the present invention and the Genbank referenced sequences.

Thus, the gaoA gene encoding the galactose oxidase enzyme from *Dactylium dendroides* is well known in the art, and the sequence has both been published and is easily accessible to one of ordinary skill in the art.

In light of the above-presented and arguments, the rejection under 35 USC 112, first paragraph is believed to be overcome and withdrawal of such is kindly requested.

35 USC 112, second paragraph

Claims 63-82 and 113-114 are rejected under 35 USC 112, second paragraph for indefiniteness. The Examiner asserts that in claims 63 and 64, the position of the amino acids is unclear because the claims do not clearly identify the referenced polypeptide. As suggested by the Examiner, claims 63 and 64 have been amended to state "which variant has a mutation in at least

[1] McPherson, M.J., Ogel, Z.B., Stevens, C., Yadav, K.D.S., Keen, J.N., and Knowles, P.F. (1992) Galactose oxidase of Dactylium dendroides., J. Biol. Chem., 267(12), 8146-8152).

one amino acid corresponding to an amino acid selected from the group consisting of S10, M70, C515, N537 and N413 of SEQ ID NO:10". Further, the amended claims have the following clarification: "a wild-type galactose oxidase having the sequence of SEQ ID NO: 10 wherein the D amino acid at position 537 is N" in the wild-type. This makes clear that SEQ ID NO: 10 differs from the amino acid sequence for wild-type galactose oxidase from *Dactylium dendroides* at only the N537 amino acid. See specification, at page 47, Table 4, and Figure 17A.

In light of the above-presented and arguments, the rejection under 35 USC 112, second paragraph is believed to be overcome and withdrawal of such is kindly requested.

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to pass this application to issue. If the Examiner believes that a telephone conversation would help advance the prosecution of this case, the Examiner is respectfully requested to call the undersigned attorney at (212) 527-7766.

Dated: August 19, 2004

Respectfully submitted,

By _____
Robert Schaffer
Registration No.: 31,194
DARBY & DARBY P.C.
P.O. Box 5257
New York, New York 10150-5257
(212) 527-7700
(212) 753-6237 (Fax)
Attorneys/Agents For Applicant

NCBI Protein

Entrez PubMed Nucleotide Protein Genome Structure PMC Taxonomy Bc

Search Protein for galactose oxidase dactylium dendroides Go Clear

Limits Preview/Index History Clipboard Details

About Entrez

Entrez Protein
Help | FAQ

Entrez Tools

Check sequence revision history

LinkOut

Related resources BLAST

Reference sequence project

Search for Genes

Clusters of orthologous groups

Protein reviews on the web

Search for full length cDNAs

Display Summary Show: 20 Send to Text

Items 1-11 of 11 One page.

☐ **1:** Q01745 BLink, Domains, Links
Galactose oxidase precursor (GAO)
gi|399532|sp|Q01745|GAOA_DACDE[399532]

☐ **2:** P06294 BLink, Links
Galactose oxidase inhibitor
gi|124243|sp|P06294|IGAO_DACDE[124243]

☐ **3:** NP_627066 BLink, Links
putative secreted protein. [Streptomyces coelicolor A3(2)]
gi|21221287|ref|NP_627066.1|[21221287]

☐ **4:** CAB65567 BLink, Links
putative secreted protein. [Streptomyces coelicolor A3(2)]
gi|6689170|emb|CAB65567.1|[6689170]

☐ **5:** A38084 BLink, Domains, Links
galactose oxidase (EC 1.1.3.9) precursor [validated] - fungus (Cladobotryum dendroides)
gi|482303|pir||A38084[482303]

☐ **6:** XEYDGD BLink, Links
galactose oxidase inhibitor - fungus (Cladobotryum dendroides)
gi|68868|pir||XEYDGD[68868]

☐ **7:** CAA77680 BLink, Domains, Links
fbfB gene [Stigmatella aurantiaca]
gi|1360139|emb|CAA77680.1|[1360139]

☐ **8:** 1GOH BLink, Domains, Links
Chain , Galactose Oxidase (E.C.1.1.3.9) (Apo Form)
gi|494054|pdb|1GOH| [494054]

☐ **9:** 1GOG BLink, Domains, Links
Chain , Galactose Oxidase (E.C.1.1.3.9) (Ph 7.0)
gi|494053|pdb|1GOG| [494053]

☐ **10:** 1GOF BLink, Domains, Links
Chain , Galactose Oxidase (E.C.1.1.3.9) (Ph 4.5)

gi|494052|pdb|1GOF| [494052]

☐ **11:** AAA16228 BLink, Domains, Links
galactose oxidase
gi|167226|gb|AAA16228.1|[167226]

Display Summary Show: 20 Send to Text

Disclaimer | Write to the Help Desk
NCBI | NLM | NIH

Jul 27 2004 06:47:37

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1992 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 267, No. 12, Issue of April 25, pp. 8146-8152, 1992
Printed in U.S.A.

Galactose Oxidase of *Dactylium dendroides*

GENE CLONING AND SEQUENCE ANALYSIS*

(Received for publication, November 4, 1991)

Michael J. McPherson‡, Zumrut B. Ogel§, Conrad Stevens¶, Kapil D. S. Yadav‖, Jeffrey N. Keen, and Peter F. Knowles

From the Department of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, United Kingdom

The *gaoA* gene, encoding the secreted copper-containing enzyme galactose oxidase, has been isolated from the Deuteromycete fungus *Dactylium dendroides*. Degenerate oligonucleotide primers were designed from amino acid sequence data for use in the polymerase chain reaction. A 1.4-kilobase DNA fragment amplified from genomic DNA was used to screen a genomic library constructed in ZAP. A strongly hybridizing clone was rescued as a pBluescript derivative, pGAO9, by *in vivo* excision. The sequence of 3466 nucleotides of pGAO9 insert DNA was determined by progressively designing sequencing primers. The translation product of the single long open reading frame matches the available galactose oxidase peptide sequence data, which represents 42% of the residues in the protein. The mature enzyme has 639 residues, which have been assigned to a 1.7-Å electron density map (Ito, N., Phillips, S. E. V., Stevens, C., Ogel, Z. B., McPherson, M. J., Keen, J. N., Yadav, K. D. S., and Knowles, P. F. (1991) *Nature* 350, 87-90). The gene lacks introns and encodes an mRNA of approximately 2.5 kilobases with three transcription initiation start points at least 324 nucleotides upstream of the translation start site. Multiple ATG codons are present between the transcription initiation region and the start of the mature protein; two in-frame ATGs could encode the initiating Met residue to give proteins with 89 or 41 residue N-terminal leader peptides. The shorter potential leader has N-terminal features characteristic of a secretion signal sequence and may also contain a pro-sequence processed by an enzyme specific for a monobasic (arginine) cleavage site, as proposed for other fungal genes. The codon bias of *gaoA* is characteristic of other filamentous fungal genes. No significant homologies exist between galactose oxidase and other protein sequences available in data bases.

Galactose oxidase (EC 1.1.3.9) is secreted by the Deuteromycete fungus *Dactylium dendroides* and catalyzes the oxidation of a range of primary alcohols, including D-galactose, to the corresponding aldehyde, with reduction of oxygen to hydrogen peroxide. Despite a wide substrate specificity, the enzyme displays remarkable stereospecificity, oxidizing only D-isomers of substrate molecules. Galactose oxidase contains one Cu(II) atom yet catalyzes a two-electron transfer reaction, implying the existence of a second co-factor. To account for this additional redox requirement, it has been proposed that the enzyme contains pyrroloquinoline quinone as a covalently bound cofactor (van der Meer *et al.*, 1989) or that an enzyme-linked tyrosine radical species is present (Whittaker *et al.*, 1989). The three-dimensional crystal structure of galactose oxidase to 1.7- Å resolution (Ito *et al.*, 1991) has no electron density corresponding to an extrinsic organic cofactor. However, the structural model reveals the existence of a novel intramolecular covalent thioether bond not previously observed in a protein and provides support for involvement of a tyrosine radical in the catalytic mechanism (Ito *et al.*, 1991). The thioether bond links the thiol group of Cys^{228} with the C_α of Tyr^{272}, one of the groups liganded to the copper, and there is a stacking interaction with Trp^{290}, which would stabilize the radical species (Ito *et al.*, 1991). The structural model also reveals extensive β-sheet secondary structure, which is consistent with the high stability of the enzyme, as shown by maintenance of catalytic activity in 6 M urea (Kosman *et al.*, 1974). Stability is also influenced by the glycosylation level of the enzyme as demonstrated by Mendonca and Zancan (1988); unusually the intracellular form of the enzyme is more heavily glycosylated and exhibits greater stability than the extracellular form.

In the present paper, characterization of galactose oxidase by peptide sequence studies, gene cloning, DNA sequencing, and transcriptional studies are reported. Various features revealed by these sequencing studies, including a long untranslated upstream sequence, 41-amino acid putative leader sequence, and a lack of *N*-glycosylation sites are discussed.

MATERIALS AND METHODS

Strains and Vectors

A culture of *D. dendroides* (Northern Regional Research Laboratory No. 2993, Peoria, IL) was kindly donated by Prof. D. J. Kosman, Department of Biochemistry, State University of New York, Buffalo, NY.

The *Escherichia coli* strains BB4 (*supE58, supE44, hsdR514p*(r_k^-, m_k^-), *galK2, galT22, trpR55, metB1, tonA*, Δ(*arg-lac*)U169 [F′, *proAB, lacI*^{q}ZΔM15, Tn10(tet^r)] and XL1-Blue (*endA1, supE44, hsdR17*(r_k^-, m_k^+), *thi-1, recA1, gyrA96, relA1*, (lac$^-$) [F′, *proAB, lacI*^{q}ZΔM15, Tn10(tet^r)] were from Stratagene. The insertional expression vector λ ZAP and pBluescript were also from Stratagene.

* This work was supported by a grant from the University of Leeds Research Fund (to M. J. M. and P. F. K.). Peptide sequencing experiments were funded by the Science and Engineering Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M86819.

‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, United Kingdom. Tel.: 0532-332595; Fax: 0532-333167.

§ Supported by the Higher Education Foundation of the Turkish Government. On study leave from the Department of Food Engineering, Middle East Technical University, Ankara, Turkey.

¶ Supported by a Medical Research Council studentship.

‖ Present address: Dept. of Chemistry, University of Gorakhpur, Gorakhpur 273001, United Provinces, India.

Purification of Galactose Oxidase

Galactose oxidase was purified from the culture medium of *D. dendroides* grown for 5 days under the growth conditions described by Tressel and Kosman (1982), except that a supplement of the trace metals was added after day 3 to stabilize the enzyme (Markus *et al.*, 1965). The purification procedure was modified from that described by Tressel and Kosman (1982) as follows.

(i) The culture medium (approximately 24 liters) was concentrated to 400 ml using a Millipore MINITAN system and then dialyzed against two changes of 10 liters of 10 mM sodium phosphate buffer, pH 7.0.

(ii) The conductivity of the dialyzed enzyme was adjusted with distilled water to be the same as 10 mM sodium phosphate buffer, pH 7.0, prior to adding 150 g of DEAE-cellulose equilibrated with the same buffer. The slurry was stirred at 4 °C for 20 min and then filtered through a Buchner funnel. The DEAE-cellulose was extracted twice further by stirring for 20 min at 4 °C with 300 ml of 10 mM sodium phosphate buffer, pH 7.0, and filtered through a Buchner funnel. The combined filtrates were concentrated to a volume of 30 ml, dialyzed for 16 h against 0.1 M ammonium acetate buffer, pH 7.2, and chromatographed on Sepharose 6B as described by Tressel and Kosman (1982). The purified enzyme typically had a specific activity of 2500 enzyme units/mg in the *o*-dianisidine-coupled assay system described by Tressel and Kosman (1982) and ran as a single band during SDS[1]-polyacrylamide gel electrophoresis.

Preparation of Copper-free Galactose Oxidase

Galactose oxidase (6 mg of protein; 3.5 mg/ml in 0.1 M ammonium acetate buffer, pH 7.2) was dialyzed against 0.01 M PIPES buffer, pH 7.0, for 16 h and then against 0.025 M PIPES, pH 7.0, containing 20 mM sodium diethylthiocarbamate for 24 h to remove the copper. Thiocarbamate was removed by sequential dialysis against three changes of 10 mM PIPES, pH 7.0, followed by three changes of water for 5 h each. The volume excess for all dialysis steps was 1000-fold. The protein was then freeze-dried and stored at −20 °C.

Carboxymethylation

Freeze-dried copper-free galactose oxidase (4.5 mg) was dissolved in 1 ml of nitrogen-saturated 0.3 M Tris-HCl buffer, pH 8.6, 6.0 M guanidinium chloride. 50 μl of a stock solution of 0.5 M dithiothreitol prepared in the same buffer was added, the protein surface was purged with nitrogen, and the sample was incubated at 37 °C for 1.5 h. 0.5 ml of a 0.5 M solution of iodoacetic acid prepared in the Tris/guanidinium solution was added, the tube was purged with nitrogen, and incubation was continued at 37 °C for 1 h. The samples were dialyzed against five changes of a 1000-fold excess of water overnight and were then freeze-dried.

Proteolytic and Chemical Digests and N-terminal Amino Acid Sequencing

Proteolytic digests with endoproteinase Lys-C or protease V8 and cleavage with cyanogen bromide were performed on 400 μg portions of the copper-free, carboxymethylated protein. The reaction conditions were as follows. Endoproteinase Lys-C digestions were carried out in 0.1 M sodium phosphate buffer, pH 7.8, containing 0.2% SDS, 8 μg of Lys-C were added, and samples were incubated at 30 °C for 12 h. V-8 protease digestions were carried out in the same buffer, 8 μg of V-8 was added, and samples were incubated at 30 °C for 1 or 7 h. Cyanogen bromide digestions were carried out in 70% (v/v) formic acid with 8 μmol of cyanogen bromide (100-fold excess over methionine residues) and incubation at 20 °C under nitrogen in the dark for 24 h. The peptide digests were separated by SDS-polyacrylamide gel electrophoresis using 15% gels (Hames, 1981). After brief staining of the gel with Coomassie Blue and destaining, the peptides were recovered by electroelution from gel slices (Sambrook *et al.*, 1989). Recovered protein was dialyzed exhaustively against water to remove traces of detergent and buffer. The peptides were freeze-dried, coupled to *p*-phenylene diisothiocyanate glass and subjected to Edman degradation on a microsequencing facility. Released amino acids were assigned following HPLC reverse phase analysis. The carboxymethylated enzyme itself was also subjected to N-terminal sequencing.

Preparation of D. dendroides Genomic DNA

D. dendroides mycelium was collected by filtration through muslin, frozen in foil packets in liquid nitrogen, and then rapidly ground to a powder with a pestle without thawing. The powdered mycelium was stored at −70 °C until required for preparation of DNA according to the spermidine-buffer method of Azevedo *et al.* (1990).

PCR Amplification of Part of the gaoA Gene

Peptide sequence data determined by N-terminal amino acid sequence analysis of mature galactose oxidase and from galactose oxidase-derived peptides were used to design oligonucleotide primers for the PCR. Oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesis instrument and were used after deprotection with no further purification. Primers were synthesized as mixtures of sequences to allow for the redundancy of the genetic code and are shown in Fig. 1. PCR amplification was performed in a final volume of 50 μl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP (Pharmacia LKB Biotechnology Inc.), 2.5 units of *Taq* polymerase (Amplitaq or Cambio type III), 100 pmol of each primer, and 0.5 μg of *D. dendroides* genomic DNA. The reaction mix was overlaid with 50 μl of light mineral oil (Sigma), and reactions were performed in a LEP PREM II thermal heat cycler. An initial denaturation step of 5 min at 95 °C was followed by 35 cycles (95 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min) with a final incubation at 72 °C for 2 min. The reaction products were separated through a 2% Nusieve agarose gel (FMC Bioproducts). The 1.4-kb DNA fragment was recovered from a gel slice by placing in a Spin-X filter (Costar), freezing at −20 °C, and then thawing and centrifuging at 13,000 × *g* for 5 min at room temperature in a microcentrifuge. The solution recovered in the tube contained the DNA that was used for subsequent manipulations without further purification.

Preparation of Hybridization Probes

Nonradioactive DNA probes for Enhanced ChemiLuminescence (ECL; Amersham Corp.) detection were labeled according to the manufacturers' instructions immediately prior to use. Radiolabeled probes were prepared by the random hexamer method of Feinberg and Vogelstein (1984) and were purified from unincorporated nucleotides by spermine precipitation (Hoopes and McClure, 1981).

Southern Blot Analysis of D. dendroides Genomic DNA

10-μg aliquots of *D. dendroides* genomic DNA were subjected to single and double digestions with the restriction enzymes *Eco*RI, *Hin*dIII, *Pst*I, and *Bam*HI. The digests were fractionated by electro-

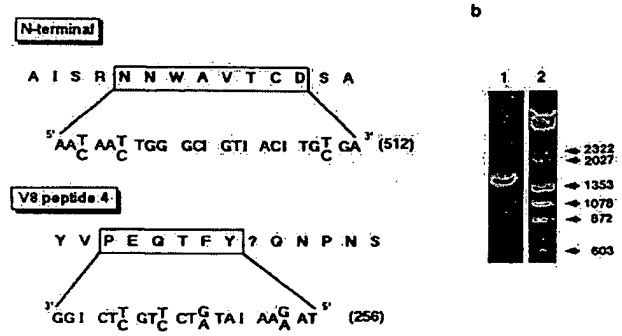

FIG. 1. **Polymerase chain reaction amplification of *D. dendroides* genomic DNA.** *a*, regions of peptide sequence used for the design of oligonucleotide primers are *boxed*. The primers were designed by back-translation of the peptide sequence and were synthesized with mixed bases or inosine at positions of base redundancy. The potential number of different DNA sequences to which the primers are complementary are shown in *brackets* after each sequence. *b*, 1.5% agarose gel stained with ethidium bromide. *Lane 1*, 1.4-kb product of PCR amplification of 0.5 μg of *D. dendroides* genomic DNA by the primers shown in *a*. Conditions for the PCR are given under "Materials and Methods." *Lane 2*, λ *Hin*dIII and φX174 *Hae*III digests. The sizes (bp) of marker fragments are indicated.

[1] The abbreviations used are: SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; kb, kilobase(s); ECL, Enhanced ChemiLuminescence; PIPES, 1,4-piperazinediethanesulfonic acid; MES, 4-morpholineethanesulfonic acid.

phoresis for 16 h (1 V/cm) through a 1% agarose gel (Sigma Type II; 20 × 15 cm) containing 1 μg/ml ethidium bromide in Tris acetate electrophoresis buffer (Sambrook *et al.*, 1989). The gel was photographed under UV illumination (365 nm) and then capillary blotted to Hybond N⁺ membrane (Amersham Corp.) according to the manufacturers' instructions.

The filter was prehybridized in 90 ml of ECL hybridization fluid in a shaking water bath for 30 min at 42 °C. Probe DNA (0.2 μg of the 1.4-kb PCR-amplified DNA) was added to the prehybridization buffer, and hybridization was allowed to proceed at 42 °C overnight. The filter was washed at 42 °C for 20 min/wash two times in a solution containing 6 M urea, 0.4% SDS, and 0.5 × SSC, two times in 6 M urea, 0.4% SDS, and 0.05 × SSC, and then finally for 5 min at room temperature in 2 × SSC. The membrane was reacted with luminol and exposed to x-ray film for 1, 10, and 50 min according to the manufacturers' instructions.

Construction of a D. dendroides Genomic DNA Library

Genomic DNA was partially digested with restriction endonuclease *Eco*RI, and the digestion products were separated through a 0.8% agarose gel containing 0.5 μg/ml ethidium bromide. Sections of gel containing DNA fragments of approximately 6–10 kb were excised, and the DNA was recovered by electroelution, phenol extraction, and ethanol precipitation (Sambrook *et al.*, 1989). A ligation reaction of 5 μl total volume containing 0.4 μg size-fractionated *D. dendroides* DNA, 1 μg *Eco*RI-cleaved and dephosphorylated λ ZAP DNA (Stratagene), and ligase buffer (66 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP) and 2 units ligase was incubated for 16 h at 15 °C. One-fifth of the ligation reaction was packaged using a Gigapack Plus packaging system (Stratagene) according to the manufacturers' instructions, resulting in a library comprising some 2 × 10^5 plaque-forming units, of which approximately 98% were recombinants, as judged by a white plaque color when plated in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and isopropyl-1-thio-β-D-galactopyranoside agar. One-half of this library was amplified to a titer of 10^{11} plaque-forming units/ml according to Sambrook *et al.* (1989).

Library Screening

Duplicate plaque lifts were taken onto Hybond N⁺ filters (Amersham plc) from six agar plates, each with approximately 8,000 plaques. Filters were screened using the ECL hybridization system (Amersham plc) using 0.25 μg of the 1.4-kb PCR-amplified DNA as probe. Following hybridization and two washes of 20 min in 6 M urea, 0.4% SDS, 0.05 × SSC, all at 42 °C, the filters were developed, and enhanced chemiluminescence was recorded on x-ray film by 1- and 60-min exposures. Positively hybridizing regions were purified to single plaques through two further rounds of screening.

In Vivo Rescue of pBluescript Recombinants

pBluescript recombinants were rescued by M13 superinfection of *E. coli* cells carrying selected λ ZAP phage. Ampicillin-resistant colonies were analyzed by PCR screening for amplification of the 1.4-kb fragment and by restriction endonuclease digestion of plasmid DNA.

Single Specific Primer PCR

To identify clones carrying additional *Eco*RI insert fragments, a single specific primer PCR approach (Shyamala and Ames, 1989) was employed in which a primer specific for the galactose oxidase gene was used in combination with a pBluescript-specific primer to direct the amplification of DNA upstream from the *gaoA*-coding region. Single plaques representing independent positive clones were transferred into a 20-μl reaction containing 20 pmol of each primer (one vector-specific and one *gaoA*-specific), 0.2 mM each dNTP, 1 × Amplitaq buffer, and 2 units of *Taq* polymerase. The reactions were performed by heating to 95 °C for 5 min, followed by 25 cycles (94 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min). Reaction products were analyzed by electrophoresis through agarose gels.

DNA Sequence Analysis

Plasmid DNA—Plasmid DNA was prepared by the alkaline lysis method (Sambrook *et al.*, 1989) with further purification by polyethylene glycol precipitation as described by Kraft *et al.* (1988). DNA sequencing reactions were performed on alkaline denatured plasmid DNA according to the protocol supplied by the U. S. Biochemical Corp. for use with Sequenase version 2.0. Reaction products labeled with α-^{35}S-labeled dATP were separated through 6% acrylamide, 7 M urea gradient gels (Biggin *et al.*, 1983). The first sequencing experiments used the PCR primers (see Fig. 1) as sequencing primers to generate data from which further primers could be designed. Cycles of sequencing and primer design were continued to allow determination of the complete gene sequence. Sequencing data were compiled using the Staden software on a Vax 11/750 mainframe computer under the VMS operating system. The predicted protein coding sequence was compared with the OWL composite protein data base (Bleasby and Wootton, 1990) by using the SOOTY and SWEEP programs (Akrigg *et al.*, 1992) which are based on the Lipman and Pearson (1985) algorithms.

PCR Products—DNA recovered from an agarose gel was denatured by boiling, quenched in ice, and allowed to anneal with the appropriate primer at room temperature (McPherson *et al.*, 1991). Sequencing reactions were performed according to the preceding paragraph.

Isolation of RNA

Frozen mycelium (400–500 mg) was collected by filtration, frozen in liquid nitrogen, and then crushed to a fine powder, which was transferred to a tube precooled in liquid nitrogen. After the addition of 0.7 ml of GuHCl buffer (8.0 M guanidinium HCl, 20 mM MES, 20 mM Na_2EDTA adjusted to pH 7 with NaOH; 2-mercaptoethanol was added to a concentration of 50 mM just prior to use) and 0.7 ml of phenol/chloroform/isoamyl alcohol (25:24:1 (v/v)), the sample was homogenized by 15 strokes of a Polytron blender (Kinematica, Switzerland). The phases were separated by centrifugation (1000 × *g*, 10 min), and the aqueous layer was extracted a further seven times with the phenol solution. RNA was recovered by precipitation with 0.2 volumes of 1 M acetic acid and 0.7 volume of cold 95% ethanol at −20 °C overnight. The pellet was washed twice in 400 μl of 3.0 M sodium acetate (pH 5.5) at 4 °C and then redissolved in diethylpyrocarbonate-treated double-distilled water, and the approximate concentration was determined by spectrophotometric assay at 260 nm (Sambrook *et al.*, 1989).

S1 Nuclease Mapping

A single-stranded DNA probe for S1 nuclease mapping was prepared by the following procedure. The 0.93-kilobase pair *Eco*RI fragment of pGAO9 (Fig. 2*c*) was subcloned into pBluescript to generate pGAO10, which was linearized at the unique *PstI* site in the polylinker before use as the template in a unidirectional PCR with an oligonucleotide primer complementary to nucleotides +518 to +533 (Fig. 3). The PCR reaction was performed in Promega *Taq* polymerase buffer containing 0.25 mM dNTPs, 4 μl (40 μCi) of α-[^{32}P]dATP, 100 pmol of primer, 15 ng of template DNA, and 2.5 units of *Taq* polymerase (Promega Biotec). The reaction mix was subjected to the following temperature regime: 95 °C, 5 min and 40 cycles of 95 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min. These conditions were selected to maximize the level of full-length radioactive transcripts rather than to optimize the specific activity of the probe. Procedures for probe hybridization and S1 nuclease digestion were those described by Greene and Struhl (1989). The sizes of DNA fragments protected from nuclease S1 digestion were accurately determined by comparison with an M13 DNA sequencing ladder.

RESULTS AND DISCUSSION

Peptide Sequence Analysis—N-terminal amino acid sequences were determined for carboxymethylated galactose oxidase and for a number of peptide fragments, including seven generated by endoproteinase Lys-C, four generated by protease V8, and one by cyanogen bromide. These data (shown in Fig. 3) provide definitive amino acid sequence data for 272 residues, which represents 42% of the 639 residues in the mature form of galactose oxidase. A further 32 residues are of tentative assignment. One residue, tyrosine 272, within a Lys-C fragment that was isolated and sequenced in four separate experiments failed to give any detectable HPLC peak. This residue is involved in a novel covalent thioether bond with cysteine 228 revealed by x-ray crystallography, and would therefore not be expected to yield a peak corresponding to tyrosine on reverse phase separation by HPLC (Ito *et al.*, 1991). Unfortunately, although it would be expected that this Lys-C fragment would be "H" shaped, with the thioether

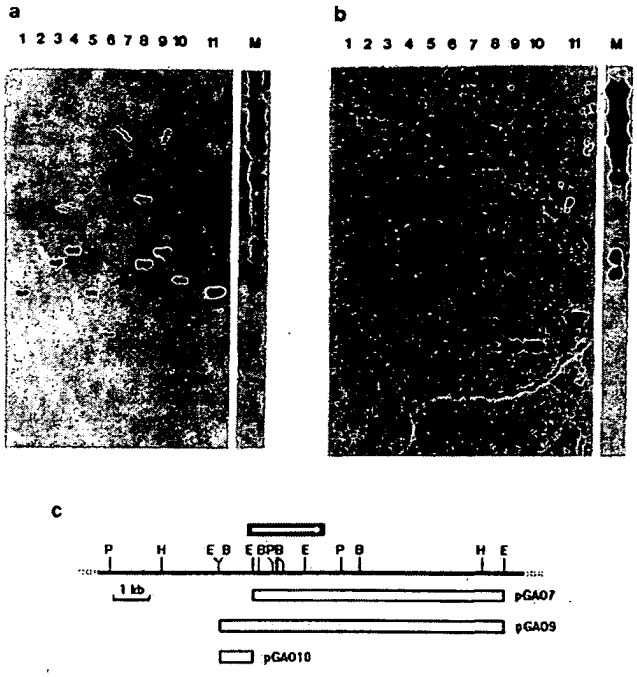

FIG. 2. **Southern blot analysis of restriction digests of *D. dendroides* genomic DNA.** *a*, photograph of the ECL image of the Southern blot probed with the 1.4-kb DNA fragment amplified from *D. dendroides* genomic DNA by the mixed oligonucleotides designed from peptide sequence data (see Fig. 1). Southern blotting was performed on a 1% agarose gel of fractionated single and double restriction enzyme digests of genomic DNA. *Lane 1*, *Eco*RI; *lane 2*, *Hin*dIII; *lane 3*, *Pst*I; *lane 4*, *Bam*HI; *lane 5*, *Eco*RI and *Hin*dIII; *lane 6*, *Eco*RI and *Pst*I; *lane 7*, *Eco*RI and *Bam*HI; *lane 8*, *Hin*dIII and *Pst*I; *lane 9*, *Hin*dIII and *Bam*HI; *lane 10*, *Pst*I and *Bam*HI; *lane 11*, positive control, approximately 0.1 ng of the 1.4-kb PCR-amplified DNA; *lane M*, λ *Hin*dIII and φX174 *Hae*III molecular size markers. The Southern blot was probed and developed according to the methods for the ECL detection system (see "Materials and Methods"). The filter was stripped of probe by heating to 95 °C for 30 min before reprobing. *Lane M* was separately hybridized to labeled λ *Hin*dIII-digested DNA and developed in parallel with the main blot. *b*, photograph of the ECL image of the Southern blot from *a* reprobed with the 1.1-kb DNA fragment amplified using primers specific for the 0.9- and 1.4-kb fragments of pGAO9 (see *c*). *c*, restriction map of the *gaoA* region of the *D. dendroides* genome. The restriction map was derived from the data shown in *a* and *b* and from analysis of the clones pGAO7 and pGAO9. *E*, *Eco*RI; *H*, *Hin*dIII; *P*, *Pst*I; *B*, *Bam*HI. The *open boxes* indicate the genomic inserts present in the three clones relevant to the present studies. The *arrow* indicates the mature protein-coding region and the direction of transcription of the *gaoA* gene.

providing a cross-link between two peptides, sequence analysis failed to detect residues from the arm containing the putative cysteine 228, perhaps due to a blocked terminal residue. However sulfhydryl titrations on the unfolded and fully reduced protein has shown that there are 5 cysteine residues in the protein (Kosman *et al.*, 1974); sulfhydryl titrations on the apoprotein reveals 1 cysteine (Kosman *et al.*, 1974), and this is confirmed by x-ray crystallography (Ito *et al.*, 1991), which further shows that the remaining 4 cysteines occur as two disulfide bridges (Cys^{18}-Cys^{27} and Cys^{515}-Cys^{518}). The gene sequence data in Fig. 3 indicate the presence of 6 cysteines, and the discrepancy between this number and the 5 cysteines identified by protein chemistry clearly supports the existence of the thioether linkage.

Design of Degenerate Primers for the PCR—N-terminal peptide sequence data from galactose oxidase and a V8 peptide were used to design oligonucleotide primers by back-translation of peptide sequence data, as shown in Fig. 1. The primers were synthesized as mixtures of oligonucleotides representing all the possible combinations of DNA-coding sequences potentially capable of hybridizing with 512 and 2048 different DNA sequences, respectively. However, the complexity of the primer mixtures was simplified by incorporating the universal base inosine at positions of 4-fold redundancy, resulting in only eight and 16 different oligonucleotide sequences in the respective primer mixes.

PCR Amplification of Part of the gaoA Gene—PCR amplification of *D. dendroides* genomic DNA with the degenerate primers produced a single product of 1.4 kb (Fig. 1*b*), which was purified and sequenced. Comparison of this translated DNA sequence with the peptide sequence data from galactose oxidase revealed identities with the last 4 residues of the peptide sequence from carboxymethylated galactose oxidase and to 52 amino acids from independently sequenced peptides generated by Lys-C, cyanogen bromide fragment, and protease V8 proteolysis. These data prove that the PCR amplification product represents part of the *gaoA* gene.

The 1.4-kb PCR-amplified DNA was labeled according to the ECL procedure and hybridized to a Southern blot of restriction digests of *D. dendroides* genomic DNA, giving the hybridization pattern shown in Fig. 2*a*. A restriction map corresponding to the chromosomal region of the *gaoA* gene, shown in Fig. 2*c*, was derived from hybridization experiments with different probe fragments (Fig. 2, *a* and *b*) and indicates that *gaoA* is a single copy gene.

Isolation of a Genomic gaoA Clone—A gene library of size-fractionated partial *Eco*RI digest fragments of *D. dendroides* genomic DNA in the insertional vector λ ZAP was screened by hybridization with the ECL-labeled 1.4-kb PCR-amplified DNA (see "Materials and Methods"). Six independent clones were *in vivo* rescued as pBluescript recombinants, and all were shown to contain *Eco*RI fragments of 1.4 and 5 kb. One clone, pGAO7, shown in Fig. 2*c*, was analyzed by DNA sequencing from the degenerate N-terminal PCR primer to confirm that the insert represented the *gaoA* gene.

Isolation of an Overlapping Clone—DNA sequencing showed that pGAO7 did not carry the start of the *gaoA* coding sequence or the upstream regulatory region. Further positively hybridizing λ clones were analyzed by single specific primer PCR (see "Materials and Methods") for the presence of cloned DNA upstream of the 1.4-kb fragment. One such clone, pGAO9, was shown by restriction analysis to contain a 0.9-kb *Eco*RI fragment in addition to the 1.4- and 5-kb fragments present in pGAO7 (Fig. 2*c*).

Contiguity of 0.9- and 1.4-kb Fragments—To prove the 0.9- and 1.4-kb *Eco*RI fragments of pGAO9 were contiguous in the genome, PCR amplifications were performed with one primer specific for the 0.9-kb fragment and one specific for the 1.4-kb fragment. The expected 1.1-kb amplification product would only be produced from genomic DNA if the 0.9- and 1.4-kb *Eco*RI fragments were contiguous and in the same relative orientation as in pGAO9. A product of the expected size, 1.1 kb, was amplified from both plasmid and genomic DNA and was ECL-labeled and used to probe a Southern blot of *D. dendroides* genomic DNA digests. The hybridization pattern (Fig. 2*b*) shows expected similarities with that produced by the 1.4-kb fragment (Fig. 2*a*), confirming that the 0.9- and 1.4-kb *Eco*RI fragments are contiguous in the *D. dendroides* genome.

DNA Sequence Determination—The DNA sequence of the *gaoA* gene was determined by a progressive sequencing strategy using plasmid DNA as template. The initial sequence reactions used the degenerate PCR primers (Fig. 1) as sequencing primers. Further oligonucleotide primers were then

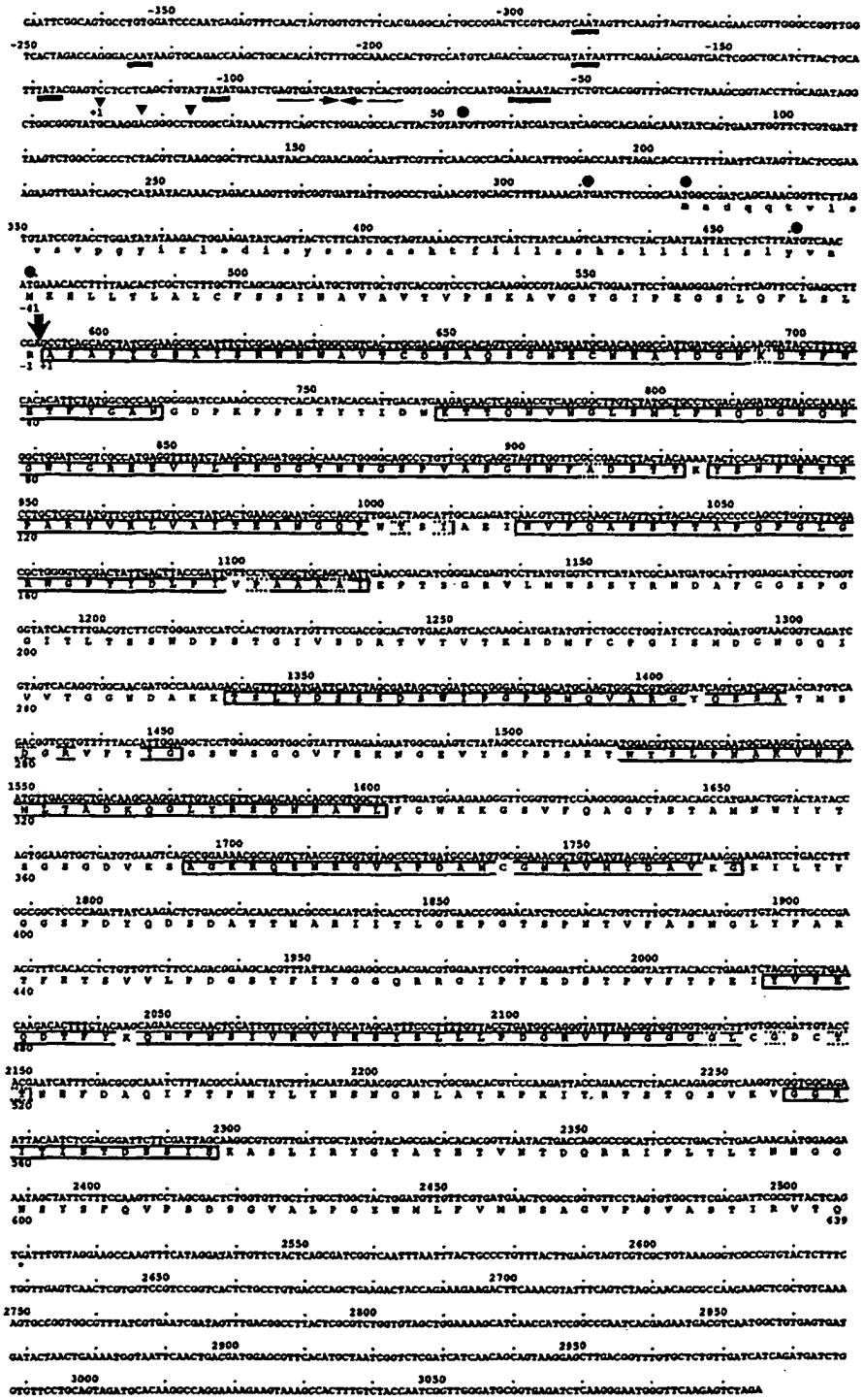

FIG. 3. **DNA sequence of the *gaoA* gene.** The transcription initiation sites are indicated by *vertical arrowheads* with possible TATA-like and CAAT sequences *underlined*. A palindromic sequence is indicated by *horizontal arrows*. The 5 ATG residues between the transcription start and the mature protein-coding region are indicated by *heavy dots*. The protein-coding region is shown in *upper case* translated as single-letter amino acid codes starting from ATG-5 contiguous with the mature protein-coding sequence. The alternative initiation site at ATG-3, which would result in the production of a protein with an addition 48 N-terminal residues, is shown (*lower case*) as a separately translated peptide. The signal sequence cleavage site is indicated by a *heavy vertical arrow*. Regions of the protein confirmed by peptide sequencing are *boxed*, with *dotted* regions indicating tentative assignments. Nucleotides are numbered above the sequence starting from position +1 as the first major transcription start point, whereas amino acids are numbered below the sequence.

designed to extend these sequence data on both strands of the DNA. This iterative process of data collection and primer design was continued until the gene sequence was complete.

The sequence of 3466 nucleotides of pGAO9 is shown in Fig. 3. One long open reading frame extends from +324 to +2507 and includes the mature protein-coding sequence (+591 to +2507) corresponding to a protein of 639 residues. For the mature enzyme, approximately 47% of the amino acids have also been determined by protein sequencing experiments, and all residues have been unambiguously assigned to a 1.7-Å electron density map of galactose oxidase (Ito *et al.*, 1991). The molecular mass of the mature protein calculated from the translated DNA sequence is 68.5 kDa, which is in very good agreement with estimates from a range of physical measurements on the enzyme (Kosman *et al.*, 1974) and from SDS-polyacrylamide gel electrophoresis analysis (data not shown). The coding region contains no introns, and there is no evidence for introns within the noncoding upstream sequence as deduced by the S1 nuclease mapping experiments discussed later.

Translation Start Site—gaoA has multiple ATG codons upstream from the most likely translation initiation site.

Examination of Fig. 3 shows that ATG-1 (+52), ATG-2 (+310), and ATG-4 (+460) would only direct the synthesis of short peptides of 13, 20, and 3 residues, respectively, and do not lie within the context of translation initiation consensus signals (Gurr *et al.*, 1987). However, both ATG-3 (+324) and ATG-5 (+468) occur in-frame with the mature protein coding sequence. ATG-3 is within the sequence CCGCAATGGC, which represents a good match to the Kozak consensus sequence CCACCATGGC. Translation from ATG-3 would result in a precursor enzyme with an 89-amino acid leader sequence. The first 48 residues of this region exhibit none of the features normally associated with a signal sequence, and an ATA (Ile) codon within this region is one of only two codons not used within the remainder of the *gaoA* coding sequence. It seems more probable that translation initiates at ATG-5, which lies closest to the mature protein coding region and would result in synthesis of a precursor with a 41-amino acid leader peptide. ATG-5 lies within the sequence TCAACATGAA, which is similar to the ATG context of a range of filamentous fungal genes and includes the important −3 A residue of the Kozak consensus (Gurr *et al.*, 1987).

Putative Leader Sequence—Pre-galactose oxidase translated from ATG-5 would have a 41-amino acid leader sequence with features analogous to those of characterized signal sequences, including (i) a positively charged N-terminal region with Lys and His adjacent to the Met and (ii) a hydrophobic uncharged region of 19 residues before 2 further basic residues. The leader sequence cleavage site defined by protein sequencing of the mature protein has an Arg at −1 and an Ala at +1. This cleavage site does not fit the predictive algorithms of von Heijne (1984, 1986), where Arg is not found at position −1, nor does it match the dibasic Lys-Arg sequence of proteins such as lignin peroxidases (Zhang *et al.*, 1991). Bussink *et al.* (1991) have recently suggested that a monobasic cleavage site in certain fungal proteins may represent the processing site for removal of a potential hexapeptide pro-peptide. These authors suggest that the precursors of the fungal proteins polygalacturonase II of *Aspergillus tubigensis* and *Aspergillus niger*, α-sarcin of *Aspergillus giganteus*, and cellobiohydrolase II of *Trichoderma reesei* show a common sequence motif ser-PRO-leu-GLU-ala-ARG preceding the mature protein, where residues in upper case are completely conserved and those in lower case are partially conserved. These sequences differ from that associated with the glucose oxidase of *A. niger* (Leu-Pro-His-Tyr-Ile-Arg), suggesting either processing by a different enzyme or by a monobasic processing enzyme with relaxed sequence specificity.

In galactose oxidase, the leader peptide of 41 residues is significantly longer than in the examples mentioned above and would most probably yield a putative pro-enzyme whose pro-sequence was significantly greater than 6 residues. However, within the 6 residues preceding the mature enzyme, Gln-Phe-Leu-Ser-Leu-Arg, there is little sequence similarity with either of the sequence motifs discussed above. The only common feature is the conserved arginine cleavage site. Although it is possible that a number of monobasic processing enzymes with differing sequence specificities exist, it is perhaps more likely, as with signal sequences (von Heijne, 1986), that a common processing enzyme with relaxed sequence specificity may recognize the Arg cleavage site of a range of pro-proteins. Alternatively, cleavage specificity could be due to structural or physicochemical characteristics of residues around the cleavage site.

Codon Usage—As for genes from other filamentous fungi, there is a marked preference for codons ending in a pyrimidine. Where codons have a purine in the third position, there is no general bias for A or G, although specific examples of amino acid bias exist. For example, GGA is used 23 times, as compared with GGG, which is used only 4 times. Two codons, ATA and CGG, are not used. It appears the codon usage pattern of this *D. dendroides* gene is more similar to those found in *Aspergillus*, rather than to those of *Neurospora*, which show extreme codon bias, particularly in highly expressed genes (Gurr *et al.*, 1987).

Glycosylation of Galactose Oxidase—Most eukaryotic extracellular proteins are modified by *O*- and/or *N*-glycosylation during their passage through the endoplasmic reticulum and Golgi, leading to greater glycosylation of extracellular than intracellular forms of the protein. In contrast, galactose oxidase appears to be deglycosylated during secretion; Mendonca and Zancan (1987, 1988) have demonstrated that intracellular galactose oxidase, which contains approximately 9% carbohydrate, has greater stability and a more restricted substrate range than the extracellular form, which contains only 2% carbohydrate. No carbohydrate was detected by the x-ray structural analysis on the extracellular galactose oxidase (Ito *et al.*, 1991), although this does not preclude its presence, since surface carbohydrate would probably be poorly ordered due to mobility and heterogeneity effects.

Examination of the translated protein sequence suggests that galactose oxidase is only modified by *O*-glycosylation, since there are no consensus Asn-*X*-Ser/Thr sequences for *N*-glycosylation (Kornfield and Kornfield, 1985). This observation conflicts with a report that tunicamycin, an inhibitor of *N*-glycosylation, affects the incorporation of [^{14}C]glucosamine and the rate of migration through SDS-polyacrylamide gel electrophoresis of intracellular but not extracellular galactose oxidase (Mendonca and Zancan, 1989). Our sequence data suggest the observed mobility difference is unlikely to be due to inhibition of *N*-glycosylation but may reflect an indirect effect on the normal processing of the enzyme and noncovalent interactions with sugars. However, the possibility that *D. dendroides* uses a different and previously uncharacterized sequence motif for *N*-glycosylation cannot be ruled out.

Transcript Analysis—Northern blot analysis revealed a transcript of approximately 2.5 kb, and the approximate location of the transcription start region was defined by a primer extension procedure involving anchored PCR (Loh *et al.*, 1989;

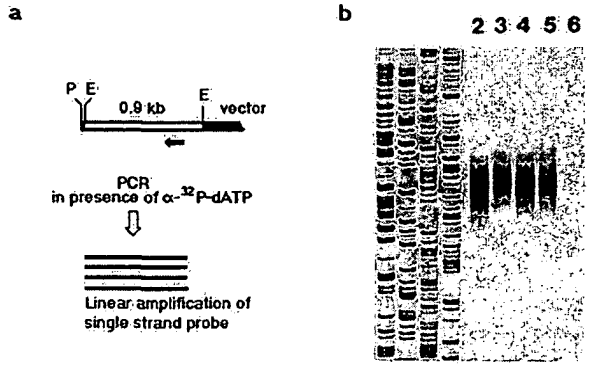

FIG. 4. **Nuclease S1 mapping of the transcription initiation region.** *a*, diagrammatic representation of the generation of single stranded probe for nuclease protection analysis. *b*, nuclease S1 protection results shown with an M13 DNA sequencing ladder to allow accurate assignment of transcription start points. *Lanes* 2–6 correspond to probe protected from nuclease S1 by RNA isolated from cultures of *D. dendroides* grown in medium containing sorbose as the sole carbon source for 2–6 days respectively. Transcript levels remain relatively constant from day 2 to day 5, with a marked loss of transcript at day 6.

data not shown). Detailed transcript mapping was achieved by S1 nuclease protection experiments using a single-stranded DNA probe prepared by asymmetric PCR in the presence of [α-^{32}P]dATP, as shown schematically in Fig. 4*a* (see "Materials and Methods").

The pattern of nuclease S1 protection of this probe by the *gaoA* mRNA is shown in Fig. 4*b*. Three major transcription start points correspond to nucleotides +1, +7, and +14 (see Fig. 3). The same pattern of protection from nuclease S1 digestion is conferred by *D. dendroides* mRNA isolated at daily intervals from 2 to 5 days of growth, which corresponds with the period of accumulation of active galactose oxidase (Kosman *et al.*, 1974). At day 6, S1 protection disappears, suggesting a loss of *gaoA* mRNA, which is probably due to transcriptional inactivation and mRNA turnover in agreement with Northern analysis of the samples (data not shown).

The S1 protection studies rule out the presence of introns within the long untranslated upstream region that represents at least 324 bases (to ATG-3) and probably 468 bases, assuming translation initiates at ATG-5. This untranslated region contains few repeat sequence elements or potential secondary structure features, and the role of this region remains to be determined. The longest untranslated upstream region previously reported for a fungal gene is 408 bases for qa-IS of *Neurospora crassa* (Huiet and Giles, 1986).

Transcription Signals—The DNA sequence upstream of the transcription start region shows a number of AT-rich regions that could represent TATA-like signals, for example ATAAAT at position −61 (Fig. 3), which is identical with the TATA-like sequence identified within the *cutA* gene of *Colletotrichum caspici*. Other possible TATA sequences are found at −105, −129, and −1172. Three matches to the CAAT box have been identified at positions −67, −236, and −292 (Fig. 3), although the first is probably too close to the transcription initiation region to be significant. It has also been noted that CAAT and TATA sequences are present at positions +322 and +366, respectively; however, these do not function as transcription start signals under the conditions used for growth of *D. dendroides* under galactose oxidase-inducing conditions (Tressel and Kosman, 1980).

Within most fungal genes, sequences similar to AAUAA, which appear to represent polyadenylation signals, are found 10–30 bases upstream from the end of the processed transcript (Gurr *et al.*, 1987). The 3′-end of the *gaoA* transcript has not been determined, and no clear polyadenylation sequence consensus exists, although a number of AU-rich regions within the untranslated 3′-region represent potential candidates for a polyadenylation signal.

Conclusions—The *gaoA* gene reveals a number of interesting features, including a long untranslated upstream region, two in-frame ATGs that could provide alternative translation start sites, a putative pro-sequence with a monobasic cleavage site, and a lack of consensus *N*-glycosylation sites, which conflicts with previously reported inhibition of *N*-glycosylation of the enzyme (Mendonca and Zancan, 1989). These features are currently the subject of further studies. In addition, the availability of the gene sequence, together with a structural model for galactose oxidase (Ito *et al.*, 1991) allows us to investigate the intriguing catalytic mechanism and other properties of this copper-containing enzyme by protein engineering studies.

Acknowledgments—We thank Veronica Blakeley and Dr. Susan Hulbert for their assistance and Dr. Sarah Gurr for stimulating and helpful discussions during these studies.

REFERENCES

Akrigg, D., Attwood, T. K., Bleasby, A. J., Findlay, J. B. C., Maughan, N. A., North, A. C. T., Parry-Smith, D. J., and Perkins, D. N. (1992) *Comput. Appl. Biosci.*, in press

Azevedo, M. S., Felipe, M. S. S., Astolfi-Filho, S., and Radford, A. (1990) *J. Gen. Microbiol.* **136**, 2569–2576

Biggin, M. D., Gibson, T. J., and Hong, G. F. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 3963–3965

Bleasby, A. J., and Wootton, J. C. (1990) *Protein Eng.* **3**, 153–159

Bussink, H. J. D., Buxton, F. P., and Visser, J. (1991) *Curr. Genet.* **19**, 467–474

Feinberg, A. P., and Vogelstein, B. (1984) *Anal. Biochem.* **137**, 266–267

Greene, J. M., and Struhl, K. (1989) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) pp. 4.6.1–4.6.13, Greene Publishing Associates and Wiley-Interscience, John Wiley & Sons, Inc., New York

Gurr, S. J., Unkles, S. E., and Kinghorn, J. R. (1987) in *Gene Structure in Eukaryotic Microbes* (Kinghorn, J. R., ed) pp. 93–139, IRL Press, Oxford

Hames, B. D. (1981) in *Gel Electrophoresis of Proteins: A Practical Approach* (Hames, B. D., and Rickwood, D., eds) pp. 1–91, IRL Press, Oxford

Hoopes, B. C., and McClure, W. R. (1981) *Nucleic Acids Res.* **9**, 5493–5504

Huiet, L., and Giles, N. H. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 3381–3385

Ito, N., Phillips, S. E. V., Stevens, C., Ogel, Z. B., McPherson, M. J., Keen, J. N., Yadav, K. D. S., and Knowles, P. F. (1991) *Nature* **350**, 87–90

Kornfield, R., and Kornfield, S. (1985) *Annu. Rev. Biochem.* **54**, 631–664

Kosman, D. J., Ettinger, M. J., Weiner, R. E., and Massaro, E. J. (1974) *Arch. Biochem. Biophys.* **165**, 456–467

Kraft, R., Tardiff, J., Krauter, K. S., and Leinwand, L. A. (1988) *BioTechniques* **6**, 544–547

Lipman, D. J., and Pearson W. R. (1985) *Science* **227**, 1435–1441

Loh, E. Y., Elliott, J. F., Cwirla, S., Lanier, L. L., and Davis, M. M. (1989) *Science* **243**, 217–220

Markus, Z., Miller, G., and Avigad, G. (1965) *Appl. Microbiol.* **13**, 686–693

McPherson, M. J., Jones, K. M., and Gurr, S. J. (1991) *PCR: A Practical Approach* (McPherson, M. J., Quirke, P., and Taylor, G. R., eds) pp. 171–186, IRL Press, Oxford

Mendonca, M. H., and Zancan, G. T. (1987) *Arch. Biochem. Biophys.* **252**, 507–514

Mendonca, M. H., and Zancan, G. T. (1988) *Arch. Biochem. Biophys.* **266**, 427–434

Mendonca, M. H., and Zancan, G. T. (1989) *Arch. Biochem. Biophys.* **275**, 130–139

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Shyamala, V., and Ames, G. F.-L. (1989) *Gene (Amst.)* **84**, 1–8

Tressel, P., and Kosman, D. J. (1982) *Methods Enzymol.* **89**, 163–171

van der Meer, R. A., Jongejan, J. A., and Duine, J. A. (1989) *J. Biol. Chem.* **264**, 7792–7794

von Heijne, G. (1984) *EMBO J.* **3**, 2315–2318

von Heijne, G. (1986) *Nucleic Acids Res.* **14**, 4683–4690

Whittaker, M. M, DeVito, V. L., Asher, S. A., and Whittaker, J. W. (1989) *J. Biol. Chem.* **264**, 7104–7106

Zhang, Y. Z., Reddy, C. A., and Rasooly, A. (1991) *Gene (Amst.)* **97**, 191–198

About Entrez

Entrez Nucleotide
Help | FAQ

Entrez Tools

Check sequence revision history

LinkOut

Related resources BLAST

Reference sequence project

Search for Genes

Submit to GenBank

Search for full length cDNAs

Display Summary Show: 20 Send to Text

Items 1-5 of 5 One page.

☐ **1:** NC_003888 Links
Streptomyces coelicolor A3(2), complete genome
gi|32141095|ref|NC_003888.3|[32141095]

☐ **2:** AL939114 Links
Streptomyces coelicolor A3(2) complete genome; segment 11/29
gi|24413773|emb|AL939114.1|SCO939114[24413773]

☐ **3:** AX232669 Links
Sequence 1 from Patent WO0162938
gi|15592663|emb|AX232669.1||pat|WO|0162938|1[15592663]

☐ **4:** Z11601 Links
S.aurantiaca fbfA and fbfB genes
gi|1360138|emb|Z11601.1|SAFBFA[1360138]

☐ **5:** M86819 Links
Dactylium dendroides (asexual stage of Cladobotryum dendroides) galactose oxidase monomer (gaoA) gene, complete cds
gi|167225|gb|M86819.1|CDOGAOA[167225]

Display Summary Show: 20 Send to Text

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1997, p. 999–1003
0066-4804/97/$04.00+0
Copyright © 1997, American Society for Microbiology
Vol. 41, No. 5

Galactose Oxidase-Glucan Binding Domain Fusion Proteins as Targeting Inhibitors of Dental Plaque Bacteria

MACIEJ LIS AND HOWARD K. KURAMITSU*

Department of Oral Biology, State University of New York at Buffalo, Buffalo, New York 14214

Received 10 October 1996/Returned for modification 15 January 1997/Accepted 25 February 1997

In order to inhibit the growth of bacteria present in the human oral cavity, a novel system which targets antimicrobial agents to dental plaque has been developed. This system involves a hybrid protein consisting of a peptide expressing the bactericidal properties of galactose oxidase (GAO) fused to the glucan binding domain (GBD) of the *Streptococcus mutans* glucosyltransferase-S enzyme. A gene encoding GAO from the fungus *Fusarium* sp. has been inserted into an *Escherichia coli* expression vector and fused to sequences encoding the GBD, which binds to the glucans synthesized by oral streptococci. Bacterial extracts expressing the hybrid protein were tested for their ability to target the GAO activity to an in vitro plaque model consisting of streptococcal cells bound to microtiter plate wells. The binding of the hybrid protein to the streptococcal cells through its GBD and the dependence of binding on the production of glucans by bacteria were demonstrated. Furthermore, killing of three different species of oral streptococci by bound hybrid protein in conjunction with the galactose-lactoperoxidase-iodide cytotoxic system has been demonstrated. These results suggest a novel strategy for controlling dental plaque formation as well as dental caries in humans.

Several chemotherapeutic agents have been utilized to inhibit the growth of cariogenic microorganisms present in dental plaque (2). Among these agents, iodine has been shown to be effective when applied topically on tooth surfaces (2). Another potent bactericidal agent, hypoiodite (OI^-), is produced by peroxidase using hydrogen peroxide and iodide as substrates (9). One potential approach for the use of hypoiodite as an antiplaque agent would be to deliver specifically to dental plaque an enzyme whose activity would ultimately lead to the local production of this antimicrobial agent. Such an antiplaque system would require coupling the effector activity to another component capable of targeting it specifically to dental plaque. The glucan binding domain (GBD) is a protein component which constitutes the carboxyl-terminal region of glucosyltransferase-S, the extracellular enzyme from *Streptococcus mutans* that catalyzes the synthesis of water-soluble glucans (4, 5). The GBD can be expressed as a separate peptide in *Escherichia coli*, and its strong affinity for glucans has been demonstrated (5a, 7). Since some of the streptococci colonizing the tooth surface produce large amounts of glucans from dietary sucrose (3), the GBD was chosen as a potential means of delivering an effector component to plaque. Glucans represent a significant component of human dental plaque and should be amenable to targeting of antimicrobial agents.

One potential antibacterial effector is the copper-containing enzyme galactose oxidase (GAO), which is produced and secreted by the fungus *Fusarium* sp. (1). This enzyme converts D-galactose and other substrates to their aldehyde forms, concomitantly producing hydrogen peroxide. In the proposed antiplaque system, after attachment of the GAO activity to surfaces colonized by cariogenic bacteria, the addition of D-galactose would stimulate local production of hydrogen peroxide. The latter could be utilized by lactoperoxidase, an enzyme present in saliva, to oxidize added iodide to hypolodite (8, 9).

In the present communication we describe the construction of a gene encoding a hybrid protein composed of GAO and GBD, its expression in *E. coli*, and the evaluation of its potential to target and kill cariogenic bacteria present in an in vitro plaque model. This system represents a potential novel antiplaque strategy for controlling dental plaque formation in humans.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and preparation of extracts. *E. coli* DH5α was used for DNA manipulations, and strain GI698 (F^- λ^- *lacI*^{q} *lacPL8* *ampC*::P_{trp}*cI*) (Invitrogen, San Diego, Calif.) was used for expression of proteins. *Streptococcus* strains *S. sanguis* 10556, *S. gordonii* Challis, *S. mutans* GS-5, and *S. mutans* GS-5ΔBCDF (12a) were used for GAO targeting assays. Strain DH5α was grown in Luria-Bertani medium; streptococci were cultivated in Todd-Hewitt broth or on tryptic soy broth (TSB) agar plates.

Expression of proteins and isolation of bacterial extracts were performed with the ThioFusion Expression System (Invitrogen) according to the manufacturer's instructions. Extracts were supplemented with Cu^{2+} ions by either directly adding $CuSO_4$ to a final concentration of 0.4 mM or dialyzing against 0.4 mM $CuSO_4$ in phosphate-buffered saline (PBS: 0.8% NaCl, 0.02% KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4).

Construction of plasmids. A 178-bp *Eco*RI-*Bam*HI DNA fragment (Fig. 1A) from plasmid pGAO9 (10), containing the 5′ end of the sequence coding for mature GAO, was subcloned into vector pUC118. One of the two *Dde*I sites on this fragment, located downstream from the codon adjacent to a cleavage site of the leader peptide, was chosen as a point of fusion with the 5′ end of the *lacZ* fragment. The 800-bp *Dde*I fragment consisting of the subcloned *gao* sequence and part of pUC118 was excised and, after the ends were filled in with the Klenow fragment of DNA polymerase I, inserted into the *Sma*I site of vector pUC118Nar (a derivative of pUC118 lacking the *Nar*I site). The resulting plasmid, pO5, was digested with *Nar*I and *Xba*I, and a *Nar*I-*Xba*I DNA fragment from pGAO9, containing the 3′ end of the gene, was inserted, generating plasmid pR3 (Fig. 1B).

The fusion of the GAO and GBD coding sequences was accomplished by isolating from pGAO9 a 495-bp *Dde*I fragment whose 3′ end is located within the last codon of the *gao* gene, filling in its ends, and inserting it into the *Hin*cII site of the vector pUC119. The sequences encoding the GBD were inserted as a *Xba*I-*Eco*RI fragment excised from plasmid pKmOZ'19GBD (12a). The generated construct containing the 3′ end of the *gao* gene in frame with the sequence encoding the GBD was removed by digestion with *Eag*I and *Bam*HI and inserted into plasmid pR3 which had been cut with *Eag*I and *Bcl*I. The resulting plasmid, pU4 (Fig. 1B), contains a complete gene encoding the GAO-GBD hybrid protein under the control of the *lac* promoter.

Plasmids pTrxFusGAO and pTrxFusGAOGBD (Fig. 1C) were constructed in two steps. Initially, the *Kpn*I-*Pst*I fragment containing the 5′ end of the *gao* gene was inserted into the expression vector pTrxFus. Then, one of the two *Pst*I fragments, containing either the 3′ end of the *gao* gene (from pR3) or that of the gene encoding the hybrid protein (from pU4), was inserted into the single *Pst*I site.

* Corresponding author. Phone: (716) 829-2068. Fax: (716) 829-3942. E-mail: Kuramits@ACSU.BUFFALO.EDU.

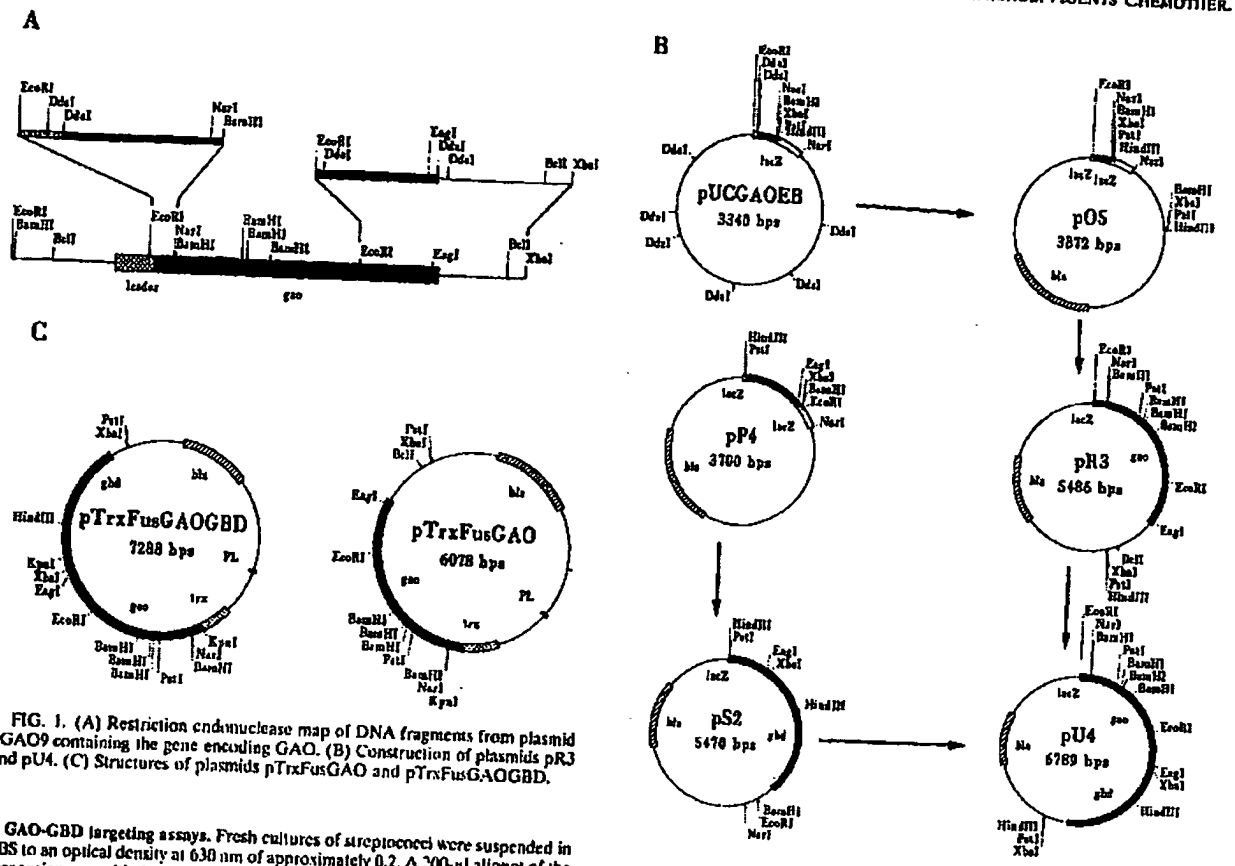

FIG. 1. (A) Restriction endonuclease map of DNA fragments from plasmid pGAO9 containing the gene encoding GAO. (B) Construction of plasmids pR3 and pU4. (C) Structures of plasmids pTrxFusGAO and pTrxFusGAOGBD.

GAO-GBD targeting assays. Fresh cultures of streptococci were suspended in PBS to an optical density at 630 nm of approximately 0.2. A 200-μl aliquot of the suspension was added to each microliter plate well, and the plate was incubated for 18 to 24 h at room temperature. The suspension, containing unbound cells, was removed from each well by vacuum aspiration, 100 μl of 10 mM sodium acetate buffer (pH 6.0) or that buffer plus 5% sucrose (0.5% in experiments performed with *S. mutans*) was added to each well, and the plate was incubated overnight at 37°C. After removal of the buffer, each well was filled with an *E. coli* extract. All extracts were adjusted to a protein concentration of approximately 1.9 mg/ml in PBS. Following incubation of the plate for 2 to 4 h at room temperature and removal of extracts, each well was washed three times with 300 μl of PBS containing 0.05% Triton X-100.

The GAO activity bound to the bacterial cells was monitored by adding 100 μl of reaction solution (5 mg of galactose, 50 μg of horseradish peroxidase, and 50 μg of *o*-dianisidine per ml in 0.1 M sodium phosphate buffer, pH 7.0) (14) and measuring the absorbance at 490 nm in a microplate reader at various time intervals.

The bactericidal activity of bound GAO was assessed by incubating the cells for 2 to 6 h at room temperature after filling each well with 100 μl of a solution containing 5 mg of galactose per ml, 10 μg of lactoperoxidase per ml, and 5 mM KI in 0.1 M sodium phosphate buffer, pH 7.0. After the solutions were removed, each well was washed once with 200 μl of PBS. The cells bound to the bottoms of the wells were covered with Todd-Hewitt broth medium, detached by scraping with a micropipette tip, plated on TSB agar, and incubated for 1 to 2 days anaerobically at 37°C.

For the dextran competition assays, extracts containing the fusion proteins were mixed with appropriate concentrations of dextran T10 and incubated for 1 h at 4°C prior to being added to the wells containing bound streptococcal cells.

Gel electrophoresis. Bacterial extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% gels. Western blots were performed with antiglucosyltransferase-S (anti-GTF-S) antibody (13). For GAO activity staining, the gels were washed for 1 h in PBS containing 1% Triton X-100 and then incubated in GAO reaction solution (14), supplemented with 0.4 mM $CuSO_4$, for 1 to 18 h at room temperature.

RESULTS AND DISCUSSION

Expression of GAO and hybrid proteins in *E. coli*. In our initial attempts to express GAO in *E. coli* and to construct GAO-GBD fusions, we used derivatives of vector pUC118 (see Materials and Methods). In both constructs (named pR3 and pU4, respectively) the GAO sequence starts from the second codon of the mature protein, and in pR3 it ends at its original termination codon. In pU4 the GAO sequence is followed by an in-frame sequence encoding the GBD. When GAO alone was expressed in *E. coli* cells, its activity was readily detected, while extracts of bacteria expressing the hybrid protein exhibited relatively low (though detectable) GAO activity (data not shown).

In order to obtain a higher level of expression of the hybrid protein, we utilized the ThioFusion expression system, in which foreign proteins can be expressed from a λ P_L promoter fused to a 12-kDa thioredoxin peptide which confers solubility to heterologous proteins. The sequences encoding both GAO and the GBD fusion proteins were inserted into the vector pTrxFus as described in Materials and Methods. The extracts of bacteria containing the resultant plasmids, pTrxFusGAO and pTrxFusGAOGBD, both exhibited significant levels of GAO activity, but the activity of the former was severalfold higher than that of the latter (data not shown) (see also Fig. 3C).

In contrast to an earlier report (11), which indicated that only enzyme containing a leader peptide sequence exhibited detectable GAO activity when expressed in a bacterial host, our findings demonstrated that active GAO in its mature form can be produced by *E. coli* cells. Furthermore, this activity may be dependent upon the additional amino acid sequences present at the N terminus of the constructs as well as supplementation of the extracts with Cu^{2+} ions.

The electrophoretic analysis of bacterial extracts (Fig. 2), involving GAO activity staining of the gels as well as Western

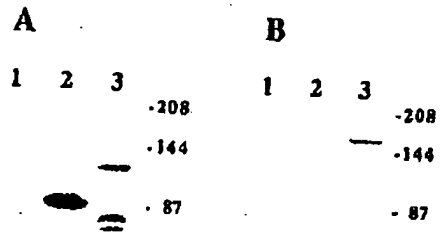

FIG. 2. GAO activity staining (A) and Western blotting with anti-GTF-S antibody (B) following SDS-PAGE of extracts of bacteria containing the following plasmids: pTrxFus (lanes 1), pTrxFusGAO (lanes 2), and pTrxFusGAOGBD (lanes 3). Molecular mass markers, in kilodaltons, are on the right.

blotting with anti-GTF-S serum (recognizing the GBD), revealed that the full-sized hybrid protein (158 kDa) did not appear to express detectable GAO activity. However, this activity was exhibited by a 122-kDa peptide visible as a distinct band on both activity-stained gels and Western blots. Two other, lower-molecular-weight bands exhibited activity following GAO staining but were not recognized by anti-GTF-S antibody. The latter peptides appear to be products of proteolytic cleavage of the fusion protein during the preparation of extracts. While it has not yet been demonstrated which part of the protein is proteolytically cleaved, a likely explanation for these results may be that the presence of an intact GBD affects the conformation of the whole protein, abolishing its enzymatic activity. In contrast, the extract expressing GAO demonstrated only a single band of approximately 90 kDa following activity staining.

The presence of the major inactive form of the hybrid protein may contribute to the marked difference in the GAO activities of the GAO and the GAO-GBD extracts. Another possible factor is the relatively low solubility of the GBD when expressed in *E. coli* from a strong promoter, which has been observed in our laboratory during previous studies of this domain.

The glucan binding capability of the 122-kDa peptide was confirmed in an experiment involving passage of the extracts through dextran T2000-Sepharose beads. The adsorbed proteins were eluted from the beads with electrophoresis loading buffer and analyzed for GAO activity following staining of SDS-PAGE gels (6). No GAO-positive protein other than the 122-kDa species was able to bind to dextran-Sepharose (data not shown).

Targeting of the GAO-GBD hybrid protein to glucan-streptococcus complexes. To initially assess the potential of the GAO-GBD hybrid protein to bind to dental plaque, we utilized microtiter plate wells covered by a layer of sucrose-grown streptococcal cells as an in vitro model plaque system. The levels of hybrid protein bound to the cellular matrix were measured as bound GAO activity in a peroxidase-coupled assay system (see Materials and Methods). Figure 3 depicts the binding of GAO and the GAO-GBD fusion protein to *S. sanguis* cells incubated with (Fig. 3A) or without (Fig. 3B) sucrose. In both cases, only negligible activity could be detected for in vitro plaque to which GAO was added, while the activity associated with the hybrid protein bound to the plaque was significant and depended on prior incubation of the cells with sucrose. The difference between the proteins is not due to the lower degree of stability of GAO, since the extract containing GAO still had severalfold higher activity than that containing the hybrid protein, as measured by direct enzyme assays following incubation at room temperature (Fig. 3C). The dependence of the binding of the hybrid protein on the presence of glucan synthesized from sucrose is consistent with our expectation that targeting of GAO activity to plaque would result from interaction between GBD and glucans. To further confirm this, we investigated the ability of dextrans (α-1,6-linked glucans) to bind to the GBD domain of the fusion protein and prevent binding of the fusion protein to the streptococcal cells. The extracts containing the hybrid protein were incubated with various concentrations of dextran T10 before cells were added. Concentrations of dextran T10 as low as 0.001% drastically reduced the targeting ability of the fusion protein (Fig. 4), while it had no effect on its galactose oxidase activity. Similar

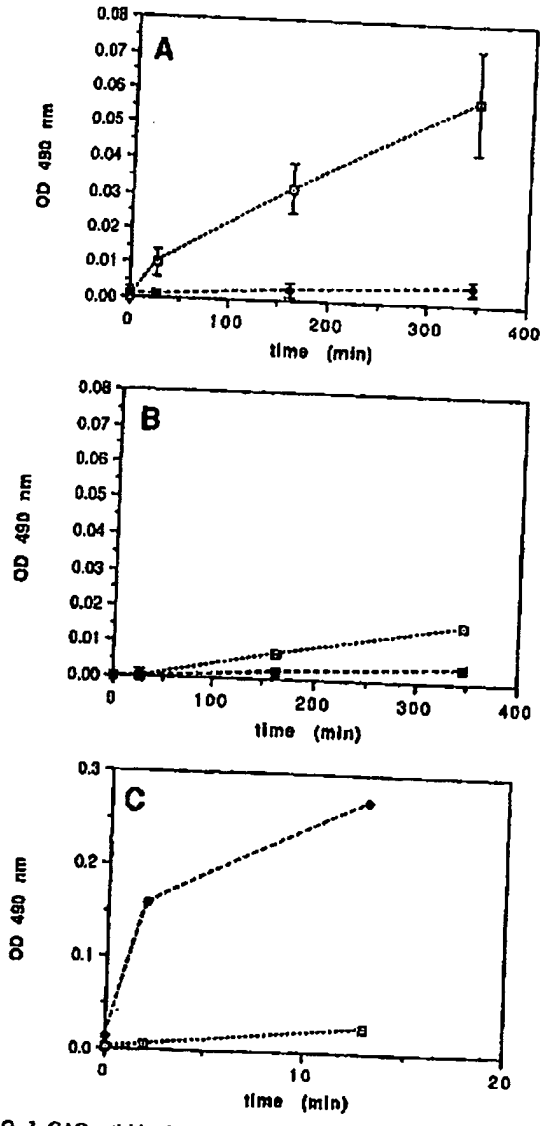

FIG. 3. GAO activities bound to *S. sanguis* cells incubated with (A) and without (B) sucrose, and direct GAO assay of extracts following incubation at room temperature (C). Symbols: □, extracts containing GAO-GBD fusion; ◆, extracts containing GAO.

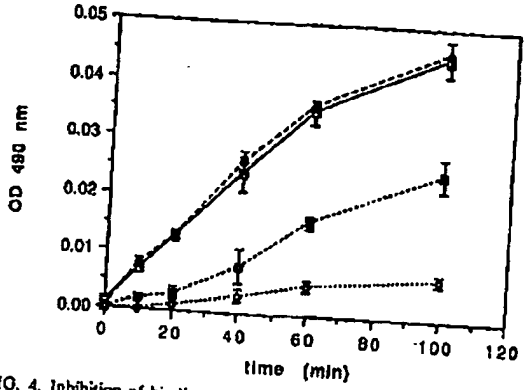

FIG. 4. Inhibition of binding of GAO activity in GAO-GBD extracts to *S. sanguis* cells by dextran T10. Concentrations of dextran are designated as follows: □, 0%; ◆, 0.0000001%; ■, 0.00001%; and ◆, 0.001%.

results were obtained when dextran T2000 was used (data not shown).

Antibacterial activity of the GAO-GBD hybrid protein. The antibacterial properties of the hybrid protein were determined as described for the targeting assays except that after the proteins were attached to streptococcal cells, the plates were incubated with a solution containing galactose, lactoperoxidase, and potassium iodide. The lactoperoxidase utilizes the hydrogen peroxide produced from galactose by GAO to convert iodide to bactericidal hypoiodite, resulting in the killing of bacterial cells (8, 9). The viability of bacteria was assessed following the plating of the plaque cells onto TSB agar plates. To *S. sanguis* cells attached to microtiter wells and incubated with (Fig. 5A) and without (Fig. 5B) sucrose were added extracts of *E. coli* carrying vector pTrxFus (bars 1 in Fig. 5) or pTrxFusGAO (bar 2 in Fig. 5A), each diluted 10-fold, or 10-, 33-, or 100-fold dilutions of extracts of bacteria containing plasmid pTrxFusGAOGBD (bars 3, 4, and 5 in Fig. 5A and 2, 3, and 4 in Fig. 5B, respectively). The viability of cells incubated with GAO alone was approximately 1 order of magnitude lower than that of the control cells. This may have resulted from nonspecific binding to the bacteria of GAO from extracts containing very high levels of the enzyme. The killing capability of extracts containing the fusion protein was dose dependent and was elevated with respect to cells previously incubated with sucrose. The killing of all bacteria in the absence of sucrose by 10-fold-diluted GAO-GBD extract (Fig. 5B, bar 2) suggests that small amounts of glucan are produced by the cells from trace amounts of sucrose in the growth medium before the cells attach to the microtiter plates. To further confirm the role of glucans in targeting the GAO-GBD fusion protein as well as to test the fusion protein on another dental plaque constituent, the targeting and killing of two strains of *S. mutans*, the wild-type strain GS-5 and its mutant, GS-5ΔBCDF, which is devoid of any GTF activity (12a), was examined (Fig. 6). Cells of both strains were incubated with sucrose prior to the addition of the extracts. While GAO alone did not affect the growth of either strain, the hybrid protein reduced the viability of only the wild-type strain, providing

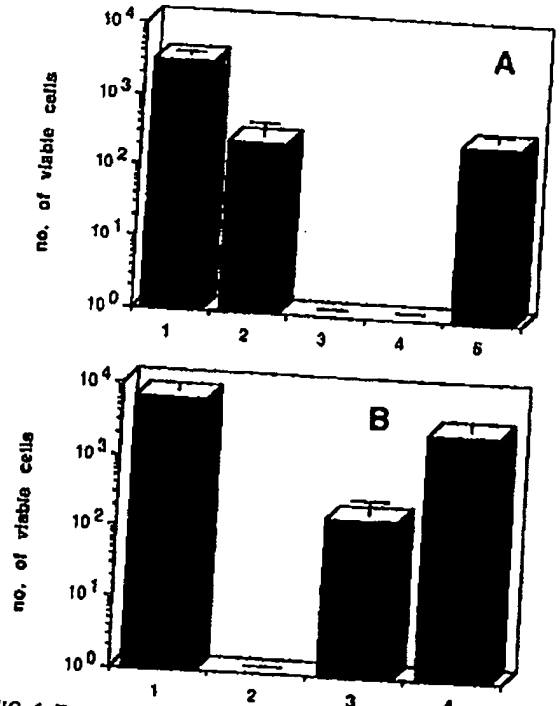

FIG. 5. Targeting and killing of *S. sanguis* cells incubated with (A) and without (B) sucrose prior to addition of extracts. (A) Cells were incubated with extracts of bacteria containing the following plasmids: pTrxFus (10-fold dilution), lane 1; pTrxFusGAO (10-fold dilution), lane 2; and pTrxFusGAOGBD (10-, 33-, and 100-fold dilutions, respectively), lanes 3 to 5. (B) Cells were incubated with extracts of bacteria containing pTrxFus (10-fold dilution; lane 1) or pTrxFusGAOGBD (10-, 33-, and 100-fold dilutions, respectively; lanes 2 to 4).

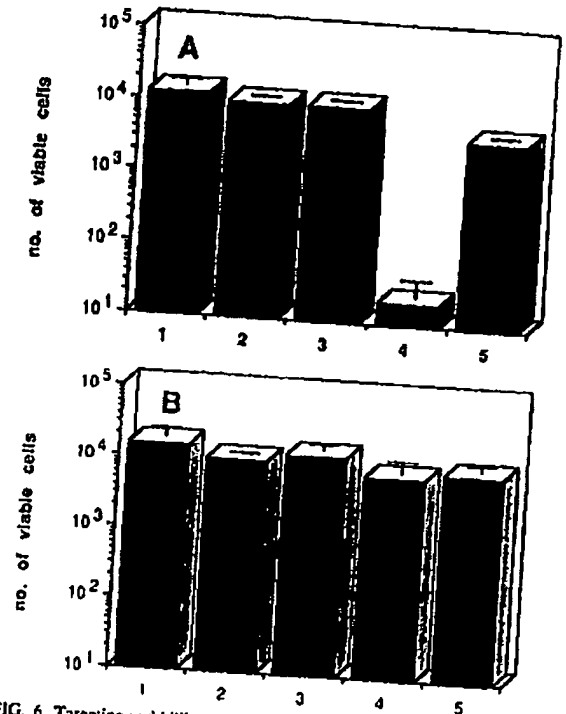

FIG. 6. Targeting and killing of two *S. mutans* strains: wild-type strain GS-5 (A) and GTF-deficient strain GS-5ΔBCDF (B). Cells were incubated with extracts of bacteria containing the following plasmids: pTrxFus (10-fold dilution), lane 1; pTrxFusGAO (10-fold dilution, lane 2); pTrxFusGAOGBD (10-fold dilution), lanes 3 and 4; and pTrxFusGAOGBD (33-fold dilution), lane 5. In lane 3, lactoperoxidase was omitted during incubation with the killing solution.

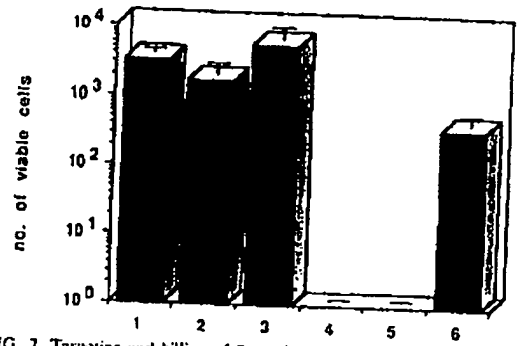

FIG. 7. Targeting and killing of *S. gordonii* cells incubated with extracts of bacteria containing the following plasmids: pTrxFus (10-fold dilution), lane 1; pTrxFusGAO (100-fold dilution), lane 2; pTrxFusGAOGBD (10-fold dilution), lanes 3 and 4; pTrxFusGAOGBD (33- and 100-fold dilution), lanes 5 and 6, respectively. In lane 3, lactoperoxidase was omitted during incubation with the killing solution.

further evidence of the critical role of glucans in targeting of the hybrid protein to streptococcal cells. This experiment also demonstrated the essential role of lactoperoxidase, whose absence in the killing solution completely abolished inhibition of bacterial growth even in the presence of bound hybrid protein (Fig. 6, bars 3). The targeting and killing of the bacterial cells by the GAO-GBD fusion protein was also confirmed with respect to another species of oral streptococcus, *S. gordonii* (Fig. 7). In this case, also, the killing of cells depended on the amount of GAO-GBD extract applied as well as on the presence of lactoperoxidase.

The GBD of GTFs from *S. mutans* has been studied extensively in several laboratories, and its ability to bind to glucans (7, 12, 15, 16) as well as to samples of human dental plaque (2a) has been well established. The data presented in this communication demonstrate the feasibility of utilizing the GBD as a means of delivering antimicrobial agents to dental plaque containing streptococcal cells producing glucans. While this antibacterial system still needs to be evaluated in in vivo animal models, the GBD, which is a component of an enzyme produced and secreted by *S. mutans* in vivo, can be expected to be stable in the oral environment. GAO, which is known for its exceptional stability, appears to be a good candidate for the second component of an antibacterial hybrid protein. Because it is not inherently bactericidal, it can be easily produced by bacterial hosts. The enzyme plays only an indirect role in antibacterial activity, leading to the production of hypoiodite, which has been demonstrated to be a highly effective bactericidal agent (8).

The present results have demonstrated targeting of a novel antibacterial system to artificial dental plaque in vitro. Improved binding and bacterial killing might be obtained by genetically engineering the GAO-GBD fusion protein gene to express a smaller GBD component, which might enhance enzyme activity without compromising targeting. In addition, such constructs need to be tested in animal model systems to examine their relative effectiveness in vivo. These results further suggest that GAO could be fused to other targeting domains for use as an antibacterial agent in other accessible regions of the body.

ACKNOWLEDGMENT

This investigation was supported by a contract from Unilever Dental Research.

REFERENCES

1. Baron, A. J., C. Stevens, C. Wilmot, K. D. Seneviratne, V. Blakeley, D. M. Dooley, S. E. V. Phillips, P. F. Knowles, and M. J. McPherson. 1994. Structure and mechanism of galactose oxidase. The free radical site. J. Biol. Chem. 269:25095–25105.
2. Caulfield, P. W., and R. J. Gibbons. 1979. Suppression of *Streptococcus mutans* in the mouths of humans by a dental prophylaxis and topically-applied iodine. J. Dent. Res. 58:1317–1326.
2a. Creeth, J. Personal communication.
3. Hamada, S., and H. D. Slade. 1980. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol. Rev. 44:331–384.
4. Hanada, N., and H. K. Kuramitsu. 1989. Isolation and characterization of the *Streptococcus mutans gtfD* gene, coding for primer-dependent soluble glucan synthesis. Infect. Immun. 57:2079–2085.
5. Honda, O., C. Kato, and H. K. Kuramitsu. 1990. Nucleotide sequence of the *Streptococcus mutans gtfD* gene encoding the glucosyltransferase-S enzyme. J. Gen. Microbiol. 136:2099–2105.
5a. Kato, C. Unpublished data.
6. Kato, C., Y. Nakano, M. Lis, and H. K. Kuramitsu. 1992. Molecular genetic analysis of the catalytic site of *Streptococcus mutans* glucosyltransferases. Biochem. Biophys. Res. Commun. 189:1184–1188.
7. Lis, M., T. Shiroza, and H. K. Kuramitsu. 1995. Role of the C-terminal direct repeating units of the *Streptococcus mutans* glucosyltransferase-S in glucan binding. Appl. Environ. Microbiol. 61:2040–2042.
8. Majerus, P. M. C., and P. A. P. Courtois. 1992. Susceptibility of *Candida albicans* to peroxidase-catalyzed oxidation products of thiocyanate, iodide and bromide. J. Biol. Buccale 20:241–245.
9. McFaul, S. J., H. Lin, and J. Everse. 1986. The mechanism of peroxidase-mediated cytotoxicity. I. Comparison of horseradish peroxidase and lactoperoxidase. Proc. Soc. Exp. Biol. Med. 183:244–249.
10. McPherson, M. J., Z. B. Ogel, C. Stevens, K. D. S. Yadav, J. N. Keen, and P. F. Knowles. 1992. Galactose oxidase of *Dactylium dendroides*. Gene cloning and sequence analysis. J. Biol. Chem. 267:8146–8152.
11. McPherson, M. J., C. Stevens, A. J. Baron, Z. B. Ogel, K. Seneviratne, C. Wilmot, N. Ito, I. Brocklebank, S. E. V. Phillips, and P. F. Knowles. 1993. Galactose oxidase: molecular analysis and mutagenesis studies. Biochem. Soc. Trans. 21:752–756.
12. Mooser, G., and C. Wong. 1988. Isolation of a glucan-binding domain of glucosyltransferase (1,6-α-glucan synthase) from *Streptococcus sobrinus*. Infect. Immun. 56:880–884.
12a. Shiroza, T., and H. K. Kuramitsu. Unpublished data.
13. Shiroza, T., and H. K. Kuramitsu. 1995. Development of a heterodimer plasmid system for the introduction of heterologous genes into streptococci. Plasmid 34:85–95.
14. Trussel, P. S., and D. J. Kosman. 1982. Galactose oxidase from *Dactylium dendroides*. Methods Enzymol. 89:163–171.
15. von Eichel-Streiber, C., M. Sauerborn, and H. K. Kuramitsu. 1992. Evidence for a modular structure of the homologous repetitive C-terminal carbohydrate-binding sites of *Clostridium difficile* toxins and *Streptococcus mutans* glucosyltransferases. J. Bacteriol. 174:6707–6710.
16. Wong, C., S. A. Hefta, R. J. Paxton, J. E. Shively, and G. Mooser. 1990. Size and subdomain architecture of the glucan-binding domain of sucrose:3-α-D-glucosyltransferase from *Streptococcus sobrinus*. Infect. Immun. 58:2165–2170.

```
LOCUS       CDOGAOA                 3466 bp    DNA     linear   PLN 04-FEB-1994
DEFINITION  Dactylium dendroides (asexual stage of Cladobotryum dendroides)
            galactose oxidase monomer (gaoA) gene, complete cds.
ACCESSION   M86819
VERSION     M86819.1  GI:167225
KEYWORDS    galactose oxidase; secreted protein.
SOURCE      Hypomyces rosellus
  ORGANISM  Hypomyces rosellus
            Eukaryota; Fungi; Ascomycota; Pezizomycotina; Sordariomycetes;
            Hypocreomycetidae; Hypocreales; Hypocreaceae; Hypomyces.
REFERENCE   1  (bases 1 to 3466)
  AUTHORS   McPherson,M.J., Ogel,Z.B., Stevens,C., Yadav,K.D., Keen,J.N. and
            Knowles,P.F.
  TITLE     Galactose oxidase of Dactylium dendroides. Gene cloning and
            sequence analysis
  JOURNAL   J. Biol. Chem. 267 (12), 8146-8152 (1992)
  MEDLINE   92235025
   PUBMED   1569070
COMMENT     Original source text: Cladobotryum dendroides DNA.
FEATURES             Location/Qualifiers
     source          1..3466
                     /organism="Hypomyces rosellus"
                     /mol_type="unassigned DNA"
                     /db_xref="taxon:5132"
                     /clone_lib="NRRL 2993"
     gene            839..2881
                     /gene="gaoA"
     CDS             839..2881
                     /gene="gaoA"
                     /EC_number="1.1.3.9"
                     /codon_start=1
                     /product="galactose oxidase"
                     /protein_id="AAA16228.1"
                     /db_xref="GI:167226"
                     /translation="MKHLLTLALCFSSINAVAVTVPHKAVGTGIPEGSLQFLSLRASA
                     PIGSAISRNNWAVTCDSAQSGNECNKAIDGNKDTFWHTFYGANGDPKPPHTYTIDMKT
                     TQNVNGLSMLPRQDGNQNGWIGRHEVYLSSDGTNWGSPVASGSWFADSTTKYSNFETR
                     PARYVRLVAITEANGQPWTSIAEINVFQASSYTAPQPGLGRWGPTIDLPIVPAAAAIE
                     PTSGRVLMWSSYRNDAFGGSPGGITLTSSWDPSTGIVSDRTVTVTKHDMFCPGISMDG
                     NGQIVVTGGNDAKKTSLYDSSSDSWIPGPDMQVARGYQSSATMSDGRVFTIGGSWSGG
                     VFEKNGEVYSPSSKTWTSLPNAKVNPMLTADKQGLYRSDNHAWLFGWKKGSVFQAGPS
                     TAMNWYYTSGSGDVKSAGKRQSNRGVAPDAMCGNAVMYDAVKGKILTFGGSPDYQDSD
                     ATTNAHIITLGEPGTSPNTVFASNGLYFARTFHTSVVLPDGSTFITGGQRRGIPFEDS
                     TPVFTPEIYVPEQDTFYKQNPNSIVRVYHSISLLLPDGRVFNGGGGLCGDCTTNHFDA
                     QIFTPNYLYNSNGNLATRPKITRTSTQSVKVGGRITISTDSSISKASLIRYGTATHTV
                     NTDQRRIPLTLTNNGGNSYSFQVPSDSGVALPGYWMLFVMNSAGVPSVASTIRVTQ"
     sig_peptide     839..961
                     /gene="gaoA"
     mat_peptide     962..2878
```

/gene="gaoA"
/product="galactose oxidase"

ORIGIN

```
       1 gaattcggca gtgcctgtgg atcccaatga gagtttcaac tagtggtgtc ttcacgaggc
      61 actgccggac tccgtcagtc aatagttcaa gttagttgga cgaaccgttg ggccggttgg
     121 tcactagacc agggacaata agtgcagacc aagctgcaca catctttgcc aaaccactgt
     181 ccatgtcaga ccgagctgat ataatttcag aagcgagtga ctcggctgca tcttactgca
     241 tttatacgag tcctcctcag ctgtattata tgatctgagt gatcatatgc tcactggtgg
     301 cgtccaatgg ataaatactt ctgtcacggt ttgcttctaa agcggtacct tgcagatagg
     361 ctggcgggta tgcaaggacg ggcctcggcc ataaactttc agctctggac gccacttact
     421 gtatgttggt tatcgatcat cagcgcacag acaaatatca gtgaattggt tctcgtgatt
     481 taagtctggc cgccctctac gtctaagcgg cttcaaataa cacgaacagg caatttcgtt
     541 tcaacgccac aaacatttgg gaccaattag acaccatttt taattcatag ttactccgaa
     601 agaagttgaa tcagctcata atacaaacta gacaaggttg tcggtgatta tttggccctg
     661 aaacgtgcag cttttaaaac atgatcttcc cgcaatggcc gatcagcaaa cggttcttag
     721 tgtatccgta cctggatata taagactgga agatatcagt tactcttcat ctgctagtaa
     781 aaccttcatc atcttatcaa gtcattctct actaattatt atctctcttt atgtcaacat
     841 gaaacacctt ttaacactcg ctctttgctt cagcagcatc aatgctgttg ctgtcaccgt
     901 ccctcacaag gccgtaggaa ctggaattcc tgaagggagt cttcagttcc tgagccttcg
     961 agcctcagca cctatcggaa gcgccatttc tcgcaacaac tgggccgtca cttgcgacag
    1021 tgcacagtcg ggaaatgaat gcaacaaggc cattgatggc aacaaggata ccttttggca
    1081 cacattctat ggcgccaacg gggatccaaa gccccctcac acatacacga ttgacatgaa
    1141 gacaactcag aacgtcaacg gcttgtctat gctgcctcga caggatggta accaaaacgg
    1201 ctggatcggt cgccatgagg tttatctaag ctcagatggc acaaactggg gcagccctgt
    1261 tgcgtcaggt agttggttcg ccgactctac tacaaaatac tccaactttg aaactcgccc
    1321 tgctcgctat gttcgtcttg tcgctatcac tgaagcgaat ggccagcctt ggactagcat
    1381 tgcagagatc aacgtcttcc aagctagttc ttacacagcc ccccagcctg gtcttggacg
    1441 ctggggtccg actattgact taccgattgt tcctgcggct gcagcaattg aaccgacatc
    1501 gggacgagtc cttatgtggt cttcatatcg caatgatgca tttggaggat cccctggtgg
    1561 tatcactttg acgtcttcct gggatccatc cactggtatt gtttccgacc gcactgtgac
    1621 agtcaccaag catgatatgt tctgccctgg tatctccatg gatggtaacg gtcagatcgt
    1681 agtcacaggt ggcaacgatg ccaagaagac cagtttgtat gattcatcta gcgatagctg
    1741 gatcccggga cctgacatgc aagtggctcg tgggtatcag tcatcagcta ccatgtcaga
    1801 cggtcgtgtt tttaccattg gaggctcctg gagcggtggc gtatttgaga agaatggcga
    1861 agtctatagc ccatcttcaa agacatggac gtccctaccc aatgccaagg tcaacccaat
    1921 gttgacggct gacaagcaag gattgtaccg ttcagacaac cacgcgtggc tctttggatg
    1981 gaagaagggt tcggtgttcc aagcgggacc tagcacagcc atgaactggt actataccag
    2041 tggaagtggt gatgtgaagt cagccggaaa acgccagtct aaccgtggtg tagcccctga
    2101 tgccatgtgc ggaaacgctg tcatgtacga cgccgttaaa ggaaagatcc tgacctttgg
    2161 cggctcccca gattatcaag actctgacgc cacaaccaac gcccacatca tcaccctcgg
    2221 tgaacccgga acatctccca acactgtctt tgctagcaat gggttgtact ttgcccgaac
    2281 gtttcacacc tctgttgttc ttccagacgg aagcacgttt attacaggag gccaacgacg
    2341 tggaattccg ttcgaggatt caaccccggt atttacacct gagatctacg tccctgaaca
    2401 agacactttc tacaagcaga accccaactc cattgttcgc gtctaccata gcatttccct
    2461 tttgttacct gatggcaggg tatttaacgg tggtggtggt ctttgtggcg attgtaccac
    2521 gaatcatttc gacgcgcaaa tctttacgcc aaactatctt tacaatagca acggcaatct
    2581 cgcgacacgt cccaagatta ccagaacctc tacacagagc gtcaaggtcg gtggcagaat
    2641 tacaatctcg acggattctt cgattagcaa ggcgtcgttg attcgctatg gtacagcgac
    2701 acacacggtt aatactgacc agcgccgcat tcccctgact ctgacaaaca atggaggaaa
    2761 tagctattct ttccaagttc ctagcgactc tggtgttgct ttgcctggct actggatgtt
    2821 gttcgtgatg aactcggccg gtgttcctag tgtggcttcg acgattcgcg ttactcagtg
    2881 atttgttagg aagccaagtt tcataggata ttgttctact cagcgatcgg tcaatttaat
    2941 ttactgccct gtttacttga agtagtcgtc gctgtaaagg gtcgccgtgt actctttctg
    3001 gttgagtcaa ctcgtggtcc gtccggtcac tctgcctgtg acccagctga agactaccag
    3061 aaagaagact tcaaacgtat ttcagtctag caacagcgcc aagaagctcg ctgtcaaaag
    3121 tgccggtggc gtttatcgtg aatcgatagt ttgacggcct tactcgcgtc tggtgtagct
    3181 ggaaaagcat caaccatccg gcccaatcac gagaatgacg tcaatggctg tgagtgatga
    3241 tactaactga aaatggtaat tcaactgacg atggagcgtt cacatgctaa tcggtctcga
    3301 tcatcaacag cagtaaggag cttgacggtt tgtgctctgt tgatcatcag atgatctggt
    3361 gttcctgcag tagatgcaca aggccaggaa aagaagtaaa gccactttgt ctaccaatcg
```

```
     3421 gttgggatgc ggtgagatct caagggaatg ggttcaagag tctaga
//
```

Disclaimer | Write to the Help Desk
NCBI | NLM | NIH

May 12 2004 07:05:19

JBC Online Have an idea for a small meeting?

HOME HELP FEEDBACK SUBSCRIPTIONS ARCHIVE SEARCH

The Journal of Biological Chemistry: Editorial Policies and Practices

The Journal of Biological Chemistry is owned and published by the American Society of Biochemistry and Molecular Biology, Inc. It is published weekly in a printed and an electronic version. Information about the JOURNAL is available on-line at: http://www.jbc.org. Its editorial policies are the responsibility of the Editor-in-Chief, the Associate Editors, and the Editorial Board under the general authority of the Publications Committee and the Council.

The JOURNAL publishes papers based on original research that are judged, after editorial review, to make a substantial contribution to the understanding of any area of biochemistry or molecular biology. Manuscripts may be submitted for consideration as Regular Papers or Accelerated Publications. The JOURNAL also publishes Minireviews, all of which are by invitation only. Authors are urged to keep the length of Regular Papers to six printed pages (24 typed pages of manuscript in double spacing including figures and tables) or less. Accelerated Publications can be no longer than four printed pages.

Accelerated Publications are intended to present new information of exceptional interest and novelty to readers of the JOURNAL. They are not intended to be short versions of Regular Papers. Because the criteria for acceptance of Accelerated Publications are considerably more stringent and the review process is expedited, an Associate Editor may judge a manuscript unsuitable without obtaining a full review.

An Accelerated Publication must be four printed pages or less, including all references, figures and tables. A four page paper is usually less than 28,000 characters plus three figures or tables (if each figure or table is less than 1/4 page). Four typed pages (1750 characters per page) with margins of one inch, double-spaced with type at least 12 points is approximately one printed page. A figure may be estimated by using a reducing photocopier to see if it can fit into a single column. Be sure to look at the smallest letter or symbol when deciding what will be legible in print. Approximately 100 references fit on a printed page (the usual four page paper has 30 or fewer references).

There is a per-page charge for all published manuscripts, currently $75 per page. Authors unable to pay page charges may apply at the time of submission for a waiver of page charges. Applications must be endorsed by a senior institutional official.

As a condition of publication, all authors must transfer copyright to the American Society of Biochemistry and Molecular Biology, Inc. Manuscripts submitted under multiple authorship are reviewed on the assumption that all listed authors concur in the submission and that the final version of the manuscript has been seen and approved by all authors.

Authors of papers published in the JOURNAL are obligated to honor any reasonable request by qualified investigators for unique propagative materials, such as cell lines, hybridomas, DNA clones, and organisms that are described in the paper. The primary data obtained from genome- or proteome-

scale analyses must be submitted for review in electronic form and, if the manuscript is accepted, must be published as Supplemental Information in JBC On-line. If computer software programs are developed and used in submitted manuscripts, the programs must be made available to the reviewer upon request. The source code or the program must be made available, either commercially or in downloadable form from the authors, if the manuscript is accepted for publication. The Editors may deny further publication rights in the Journal to authors unwilling to abide by these principles.

All manuscripts submitted to the JOURNAL are reviewed critically. It is the responsibility of the Editor-in-Chief, the Associate Editors, and the Editorial Board to determine their suitability for publication. After receipt of a manuscript by the Editor, it is sent to an Associate Editor who usually assigns it to a member of the Editorial Board. The Board member then makes a definitive recommendation for acceptance, revision, or declination based on the scientific merit and technical quality of the studies reported. Referees may be consulted when additional expertise is required; however, decisions on manuscripts are often made on the basis of a single review.

All Board members and referees who review a manuscript remain unknown to the authors. Every manuscript is treated by the Editors and referees as privileged information, and they are instructed to exclude themselves from review of any manuscript that might involve a conflict of interest or the appearance thereof.

On submission of a manuscript, authors are urged to suggest potential reviewers who have not seen the manuscript before submission, especially members of the Editorial Board.

Authors may request disqualification of a few potential reviewers, but not an Associate Editor. Only those individuals who would represent a direct conflict of interest should be excluded. Authors cannot make an extensive or blanket disqualification of a group of possible reviewers, e.g., all potential competitors in one or more industrial laboratories or academic departments. Requests to disqualify reviewers will be honored at the discretion of the Associate Editor who handles the review.

The primary criteria for judging the acceptability of a manuscript are its originality and scientific importance. Manuscripts judged lacking in these respects will be declined, even if the experimental work appears technically sound. This policy permits declination of a manuscript solely on the Editorial Board's judgment that the studies reported are not sufficiently original or important to merit publication in the JOURNAL. If a manuscript is not recommended by the reviewer for publication, the Associate Editor can decline it without further review. In some instances, the Associate Editor may also seek a second opinion from another reviewer but is not obligated to do so.

Guidelines for Editorial Decisions

The members of the Editorial Board use a set of flexible guidelines, set forth below, to assist them in making editorial decisions.

General Criteria

The primary criteria for judging the acceptability of a manuscript are originality and scientific importance.

Manuscripts failing to deal with biological processes at the biochemical or molecular level are usually inappropriate for the JOURNAL. In the absence of novelty and biochemical significance, medical relevance or pharmacological potential alone will not be considered sufficient to justify publication.

Criteria in Specific Areas

Manuscripts of the following types will be declined without full review unless additional information in the manuscript contributes sufficient biochemical insight to make the manuscript highly significant.

Methodology

Manuscripts that report development of a new technique without using the method to obtain novel and significant biochemical insights.

Sequencing and cloning

Manuscripts reporting an amino acid or nucleotide sequence, or the cloning and expression of a gene, if the same protein or gene from another tissue or organism has already been described.

Manuscripts reporting the cloning and sequencing of a novel gene without providing experimental evidence for its biological function.

Enzymology

Manuscripts describing just the purification and/or characterization of an enzyme or protein if the same enzyme or protein has been described from another tissue or organism.

Protein-protein Interactions

Manuscripts that describe biochemical analyses of protein-protein interactions or results of two-hybrid screens, co-immunoprecipitation, or related assays without providing information about the functional consequences of the interactions.

Manuscripts that rely solely on the use of protein over-expression in transfected cells or the use of recombinant proteins to demonstrate protein-protein interactions, without providing evidence that such interactions occur between protein partners expressed from the endogenous genes in relevant cell lines or tissues.

Transcription

Manuscripts that focus on identifying promoter regulatory elements and the proteins that bind them without providing new insights into molecular mechanisms of gene expression or transcription factor function.

Manuscripts that simply identify methylated sites in a gene or promoter, or demonstrate that the methylation status of a gene correlates with gene expression, without providing novel information about how methylation is controlled or how methylation controls gene expression.

Regulation of metabolism and gene expression

Manuscripts that merely describe the effects of agents such as drugs, hormones, cytokines, or the effects of the state of differentiation on an "end point" (enzyme activity, protein level, mRNA abundance, or descriptive aspect of a cellular response).

Manuscripts in which reagents are assumed to act specifically without a suitable demonstration or reference documenting their specificity.

Manuscripts that rely solely on the use of pharmacological agents to define a biochemical process.

Post-translational modification

Manuscripts describing modification of a protein by a well-established process such as glycosylation, phosphorylation, fatty acylation, or prenylation without showing the biological or biochemical significance of the modification or providing novel insights into the mechanism of the modification process.

Glycobiology

Manuscripts that report the structure of an oligosaccharide not differing substantially from an oligosaccharide that was described earlier for another glycoconjugate.

Mutational analysis of proteins

Manuscripts reporting that mutation of a protein alters its function without providing clear evidence about the mechanism by which the function is altered.

Transgenics and knockouts

Manuscripts that use transgenes or knockouts to confirm results reported previously in model systems without adding new mechanistic insight into the processes involved.

Manuscripts that report generation of transgenic or knockout mutants that lack demonstrated phenotypes and/or that fail to provide new insights into biochemical processes.

Functional Genomic and Proteomic Analysis

Manuscripts that describe genome- or proteome-scale functional analysis by differential display, microarray, mass spectrometry, or other methods without providing novel insight into a biochemical process or its regulation.

Bioinformatics

Manuscripts describing computational analyses without providing novel insights into structure, function or regulation.

Studies on Cell Cycle and Apoptosis With Flow Cytometry

Manuscripts describing studies of either cell cycle or apoptosis that use inadequate FACS analysis procedures (e.g. single parameter DNA histograms of a single time point) to establish biological states.

Last Updated: 02 April 2003

Copyright © 2003 by the American Society for Biochemistry and Molecular Biology.

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☑ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☑ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.